

Ligand Interactions of Nanoparticles and Strategies to Tune Distribution



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Dedicated to my family.

*Forschung ist, zu sehen, was schon jeder gesehen hat
und zu denken, was noch niemand gedacht hat.*

Albert Szent-Györgyi

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Chapter 1

New Perspectives on the Lock-and-Key Model in the Context of Ligand-Mediated Nanoparticle-Cell Interactions

Introduction

Abstract

When nanoparticles are functionalized with ligands in the context of active targeting strategies, the metaphor of the lock-and-key model is frequently employed to describe the interactions with their target sites. Thereby it remains disregarded that the metaphor originally referred to monovalent interactions, specifically to the interaction between one ligand, “key”, with one binding site, “lock”. In this review, we critically examine the limitations of applying the metaphor to multivalently binding nanoparticulate systems, which carry more than one “key” on their surface. We highlight the aspects where the model is overly simplistic in capturing the complexity of the nanoparticulate interactions with the host cells and provide perspectives how alternative interpretations of the “keys” and “locks” and their interactions could be conceptualized. Both, the ligand-functionalized nanoparticle surface and the cellular structures, which exhibit partially adaptive behavior towards the nanoparticles, are examined. Thereby we provide insights beyond the “lock” and on cellular response processes. With this review, we aim to contribute to a deeper understanding of the interactions between ligand-functionalized nanoparticles, recognized for their substantial potential in diverse applications, and their target sites. We believe that achieving a more profound understanding is essential for optimized and rational design approaches.

Introduction

A decisive step in advancing nanoparticles for specific applications is the functionalization of their surface with bio-recognition molecules (e.g. antibodies, small molecules, and peptides),^[1,2] also referred to as active targeting.^[3,4] In contrast to passive targeting concepts, which are based on an accumulation of nanoparticles in the tissue due to the EPR (enhanced permeability and retention) phenomenon,^[5–7] the functionalization of the nanoparticle surface is intended to increase the probability for a specific and selective nanoparticle-cell contact and therefore to improve the target/off-target ratio.^[8–10] Molecular binding interactions between the bio-recognition molecules, ligands, on the nanoparticle surface and the cell receptors are designed to mediate the first contact between the particles and the target site and to promote their adhesion to the cell surface.^[8] Subsequently, these interactions are crucial for the uptake of the nanoparticles in the cell and following downstream processes.^[11,12] A commonly used analogy to describe specific interactions between ligands and receptors is the lock-and-key model, which traces back to Emil Fischer.^[13,14] Thereby the “key” represents the ligand and the “lock” the receptor. The model is also frequently transferred to the interaction between ligand-functionalized nanoparticles and their target receptors to articulate the objective of developing systems that exhibit specific and ideally, selective binding capabilities.^[15]

Due to their scaffolds and large surface area, nanoparticles can typically carry multiple copies of ligands, “keys”, thereby exhibiting the capacity to form numerous bonds with target cells.^[16–18] The ligands are mostly attached to the nanoparticle surface via linkers, which may consist of polymers with different chain length and varying degrees of flexibility.^[15,19] The multivalent character of the binding enhances the relative affinity compared to the corresponding monovalent interaction^[20,21] and compensates for the loss of affinity of the individual ligands upon attachment to the nanoparticle surface.^[22,23] It thus augments the nanoparticle adhesion to the target site.^[24] The accumulated binding strength of the cooperative interactions of the individual ligands is referred to as the avidity of the nanoparticles^[17,25] and commonly used as a measure for favorable particle membrane interactions and their specificity.^[26] A related parameter is the binding affinity constant, also termed equilibrium dissociation constant, K_D value. It measures the strength of a single reversible interaction between a ligand and its receptor, and is defined as the quotient of the rate constant of detachment, k_{off} , and the binding rate constant, k_{on} .^[27–29] When applied to nanoparticulate systems, the K_D value serves as a measure of the binding affinity of surface ligands for their receptors.^[19] In contrast to the avidity, however, the K_D value of a nanoparticle is independent of the number and arrangement of binding sites (receptors) on the cell surface.^[30] Due to the multivalency of the interaction, the residence time of nanoparticles on the cell surface significantly increases after initial adhesion. k_{off} is consequently several orders of magnitude lower than for a monovalent interaction.^[11,31] This trend cannot be extended to k_{on} , which as describing the specific binding rate, considerably affects the kinetics of binding.^[21] The specific attachment rate to the cell surface is not correlated to the cooperativity and multivalency of the system.^[31] Regardless of the binding strength, the formation of

the multivalent binding can proceed very slow. Consequently, solely increasing the avidity of the system may not be a reliable strategy for increasing the efficiency of multivalent nanoparticles.

As multivalent bonds, nevertheless, offer a substantial advantage in binding selectivity over monovalent bonds,^[17,32] multivalent binding behavior is ubiquitous in nature, such as for example in interactions between antibodies and antigens^[30] and between viruses and cells.^[33,34] Viruses are equipped with spikes on their surface that bear binding entities, permitting them to attach to host cell receptors and achieve infectivity towards target cells.^[35] Multivalent binding patterns^[33,36] enable (super-)selectivity, allowing for the distinction between target and off-target cells, which often differ only by small variations in receptor expression levels.^[17,37] In viruses, the advantages of multivalent systems regarding target specificity and selectivity are evolutionary optimized and adapted to their cellular counterparts. Their example demonstrates the significant potential that lies in the enhancement of multivalent systems, making them ideal role models for the design of ligand-functionalized nanoparticles.^[38–40] Based on the lock-and-key model and inspired by viruses, complex active targeting concepts have been developed, partially aiming to achieve target selectivity through the sequential presentation of more than one type of ligand.^[38,41,42] A current overview of these strategies and how they determine the nanoparticles' fate in vivo has recently been published by our group.^[43] Nevertheless, achieving targeting efficiency for nanoparticles that is comparable to that of viruses remains a highly sought-after goal that has not yet been accomplished. It is therefore worthwhile to take a closer look at the nanoparticle-cell interactions to better understand the underlying mechanisms and pitfalls and to assess whether they are sufficiently characterized by the lock-and-key model.

In the present review, we aim to discuss the critical aspects influencing the specificity and selectivity of ligand-functionalized nanoparticles towards their target cells and how they are captured by the lock-and-key metaphor. We take a closer look at the physiological response that the ligand-mediated binding of nanoparticles has on target cells. Thereby we identify binding motifs, which are not encompassed by the current understanding of the lock-and-key model. We emphasize the nanoparticle concentration on target cells and the associated cooperative effects between nanoparticles as a significant factor influencing their binding behavior. Our analysis considers both binding and anti-binding effects mediated by active targeting concepts.

1. Selectivity in the Lock-and-Key Model

The target/off-target ratio and thus the targeting efficiency of ligand-modified nanoparticles is determined by the selectivity and specificity of their multivalent interactions.^[10] For this reason, the selection of appropriate ligands, “keys”, for decorating the nanoparticle surface has been extensively studied. A multitude of design approaches have been tested to optimize the nanoparticles’ surface architecture.^[44–46] To achieve (super-)selective binding, distinguishability between cells, based on the expressed receptor types, “locks”, and on minute differences in receptor expression levels, is required.^[47] Three critical parameters were identified as having significant impact on the selectivity of multivalent bonds and as being transferable across all design approaches (Figure 1): first, the flexibility of the linkers used to connect the ligands to the nanoparticles’ surface, second, the ligand density (valence), and third, the binding strength between the ligands and the target sites.^[17,32]

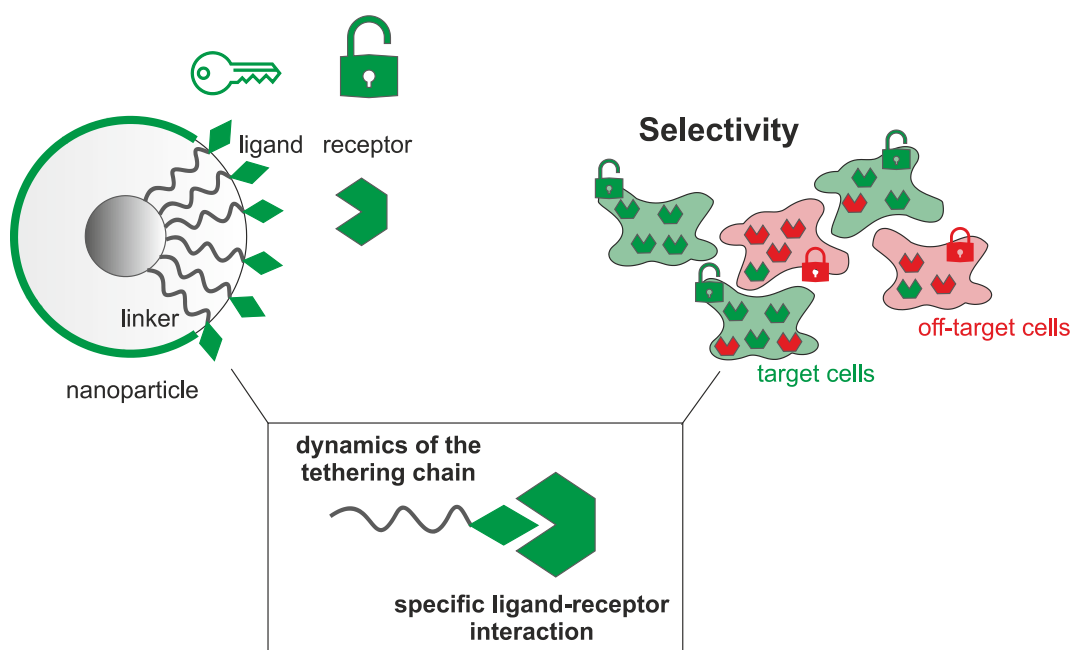


Figure 1 Selectivity between ligand-functionalized nanoparticles and target cells according to the lock-and-key model. The binding selectivity is influenced by the flexibility of the linker chains, the ligand density (valence) and the binding strength between the ligands and the target sites. The three factors are closely intertwined: Both, the specific ligand-receptor pair interaction and the dynamics of the tethering chain contribute to the overall range, rate and ultimate strength of the bond formation.

1.1 Linker Flexibility

To be accessible for interactions with receptors, ligands must possess sufficient flexibility and mobility in their spatial arrangement on the nanoparticle surface.^[15,48] Ligands, which are coupled directly to the surface of nanoparticles without interposition of linker chains, might lack the required flexibility, which translates into insufficient lock-and-key interactions.^[49] In general terms, selectivity in binding is therefore improved by opting linkers that allow a high degree of flexibility in orientation of the overall linker-ligand conjugate.^[17] Conversely, regarding the steric properties of the linkage between the

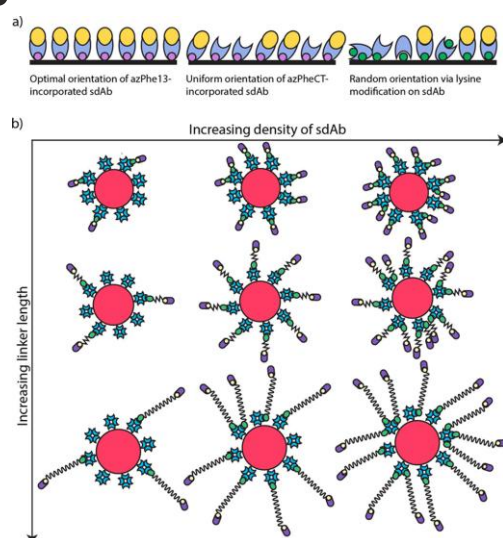
ligand unit and the linker chain, Liese and Netz^[50] identified the angular restriction as a crucial parameter for increasing binding affinity (Figure 2A). They defined the angular restriction factor of a multivalent system, ω_{LU}^n , as the ratio of the angular restriction of the ligand bound to a linker chain relative to that of the unbound, free, ligand. Their molecular model revealed that with decreasing ω_{LU}^n , meaning the more the ligand's orientation relative to the linker chain is restricted, the multivalent dissociation constant, K_D , is reduced.^[50] This results in an increased binding affinity of the multivalent system. An optimization of the nanoparticle design is also required in terms of the chain length of the linkers. On the one hand, longer linker chains allow a higher degree of flexibility and can therefore enable an optimal orientation of the ligands towards the receptors. On the other hand, they hold the potential to collapse, thus unfavorably influencing the steric orientation of the ligands or shrouding them.^[19,51] For the commonly utilized polymer chains, their degree of polymerization (chain length), together with their grafting density on the nanoparticle surface,^[52] and the quality of the surrounding solvent are fundamental determinants for their geometric structure.^[52–54] Based on these three factors, linear polymers can adopt either mushroom or brush conformation^[55] and thus rather burying the ligands or presenting them on the surface.^[56,57] Furthermore, individual linker chains experience inhomogeneous stretching and consequently exhibit varying lengths,^[58] which is favored for entropic reasons.^[52] This affects the effective ligand density on the surface of the nanoparticles and consequently the accessibility of ligands to receptors. Several studies have thus shown that shorter linker chains improve the targeting efficiency of nanoparticulate systems.^[56,59,60] The potential inherent in optimizing ligand orientation and linker length was also demonstrated by Yong et al.^[19] (Figure 2B and C). They used anti-EGFR single-domain antibody-functionalized quantum dots (Q-dots) to target epidermal growth factor receptor (EGFR)-positive cells. By incorporating a synthetic amino acid within the structure of the antibody, they were able to achieve site-specific protein conjugation. This enabled them to link the ligand in defined orientations. The antibody was coupled to the Q-Dot surface using linkers composed of dibenzocyclooctyne (DBCO)/polyethylene glycol (PEG)/biotin conjugates with different PEG chain lengths (no PEG, 4 PEG units, 12 PEG units). The optimization of the ligand orientation resulted in an 8-fold increase in the binding affinity, while the optimization of the linker length led to an at least 2-fold increase.

A Flexibility

a) linker-ligand conjugate b) linkage ligand unit - linker chain



B



C

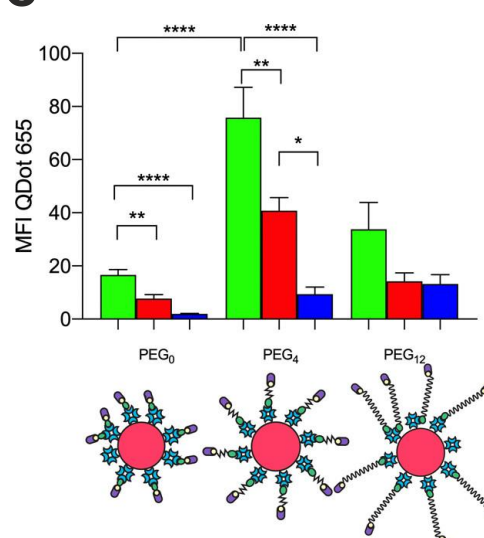


Figure 2 Linker flexibility, length and conformation and ligand orientation as critical factors for the ligand-receptor interaction. (A) The high flexibility of the (a) linker-ligand conjugate improves the selectivity of the bond formation. Conversely, a higher degree of restriction in the (b) linkage between the ligand unit and the linker chain contributes to an increased binding affinity. (B) Reprinted with permission from Yong et al.^[19] Copyright (2025) American Chemical Society. (a) Orientations of the anti-EGFR antibody relative to a solid surface. (b) Q-dots functionalized with varying antibody densities coupled to linkers with different degrees of flexibility. (C) Reprinted with permission from Yong et al.^[19] Copyright (2025) American Chemical Society. Mean fluorescence intensity (MFI) of antibody-functionalized Q-dots on EGFR-positive target cells. Linker chains with 4 PEG units (PEG₄) significantly improved the cell binding of Q-dots carrying optimally oriented antibodies (green). Uniformly on the Q-dot surface oriented (red) and randomly oriented (blue) antibodies are depicted for comparison.

1.2 Ligand Valence

The second crucial factor influencing binding selectivity is the ligand valence, or ligand density, on the nanoparticle surface. The ligand density has previously been discussed as a critical parameter for in vitro and in vivo targeting in a read-worthy review by Alkilany et al.^[35] The authors emphasize the impact of ligand density on both, in vitro and in vivo targeting efficiency, noting that the optimal ligand density may vary between these two conditions. Fundamentally, a sufficient ligand valence

must be ensured to initially mediate a stable interaction with the cell surface.^[61,62] Li et al.^[63] showed in a study on oligonucleotide-functionalized dendrimers that a threshold ligand valence must be exceeded to form high avidity interactions with slow dissociation from the target surface (Figure 3). Using nano adhesive dynamics Wang et al.^[24] established that nanoparticles, which are weakly bound with low valence to the cell surface, tend to detach over time. Meanwhile, the remaining nanoparticles, that bound with higher valence, form progressively more bonds, resulting in an overall increase in the mean bond number. Consequently, they proposed that the K_D value of multivalent systems should not be considered a constant as it decreases following a power law relationship with time. Their results indicate a time-dependent differentiation and progression towards higher valence binding.^[24] The relationship between the ligand valence on the nanoparticle surface and the selectivity of their binding, however, is highly non-linear: With an increase in the ligand density, the selectivity first rises and then drops again.^[17,64] The same applies for the avidity of nanoparticles, which may alternatively reach a plateau after initial increase.^[17,19,35]

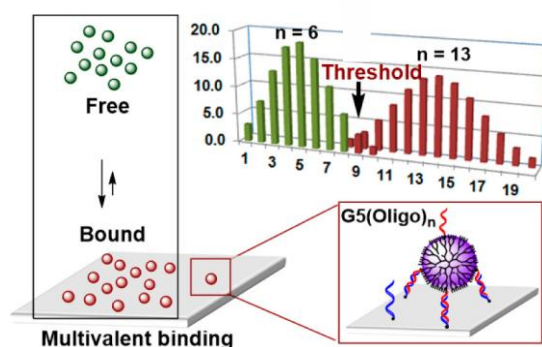


Figure 3 Ligand valence as a critical parameter for multivalent binding. Reprinted with permission from Li et al.^[63] Copyright (2025) American Chemical Society. A threshold ligand valence needs to be exceeded for multivalent systems to establish multivalent binding to target-receptor positive surfaces.

Consequently, the nanoparticle design does not gain advantage from excessive surface coverage with ligands; rather, several drawbacks are associated with high ligand densities.^[10,35,65] Steric hindrance between densely packed and overlapping polymer chains can influence the ligand flexibility and thus hinder receptor binding.^[8,19,52] Reaching a certain ligand density, the increase in binding free energy occurs at a slower rate compared to the steric repulsion of the targeted receptors, making further binding events less attractive.^[64] Additionally, a higher ligand density leads to an intensified competition for a limited number of receptors on the host cell surface and to receptor depletion.^[11,62] A high level of ligand functionalization can furthermore influence the recognition by the immune system, thereby contributing to a faster clearance from systemic circulation.^[15,35,56,66] In this regard, viruses are evolutionarily optimized.^[35] Their spike density represents the “sweet spot” to minimize immune system recognition while maximizing their infectivity.^[35] In direct comparison, nanoparticles are on average functionalized with a tenfold higher ligand valence on their surface,^[35]

which certainly weakens their cell selectivity.^[67] In a recently published study, we demonstrated that only a minority of the ligands coupled to the nanoparticle surface are actually involved in specific ligand-receptor interactions.^[67] This highlights the significant potential that remains in optimizing the ligand valence. Most important, the ligand valence influences the binding strength, which is the third control parameter to modulate the nanoparticles' selectivity.^[17]

1.3 Binding Strength

The binding strength of functionalized nanoparticles can be modulated in two ways: either by selecting ligands with initially low affinity to the target or, alternatively, by reducing the number of ligands on the nanoparticle surface.^[30] Both addresses those facets of avidity that depend inherently on the nanoparticle system and can thus be modulated design-wise.^[10] Nanoparticles, which exhibit strong avidity for the targeted receptors, are at risk of interacting also with off-target cells that express the respective receptors at lower level.^[10,68] Consequently, nanoparticles with lower avidity frequently show higher target selectivity.^[10,64,69] In this case, a sufficient avidity of the system is linked with a characteristic of the target cells, specifically their higher target receptor densities.^[30] However, the efficiency of biomedical nanoparticles is often not solely determined by their binding to the target receptors, but also their successful internalization into the cell. Unlike adhesion to the cell surface, which increases partially linear the ligand affinity, the internalization of nanoparticles appears to follow a “yes” or “no” pattern.^[70] Groza et al.^[70] utilized polyvalent virus-like particles functionalized with green fluorescent protein (GFP) to prove that an adhesion energy threshold needs to be surpassed to induce membrane deformation and endocytosis of the nanoparticles (Figure 4). The bending energy required for wrapping must be compensated by the adhesion energy,^[66] which makes a sufficiently high avidity of the nanoparticles necessary. Other studies have revealed that the ligand valence influences the uptake mechanism, e.g. clathrin- or caveolin-mediated endocytosis, of nanoparticles into cells.^[71–73] This could be potentially explained by the fact that the proteins, clathrin or caveolin, facilitate the nanoparticle binding and thus contribute to the overall binding energy.^[66]

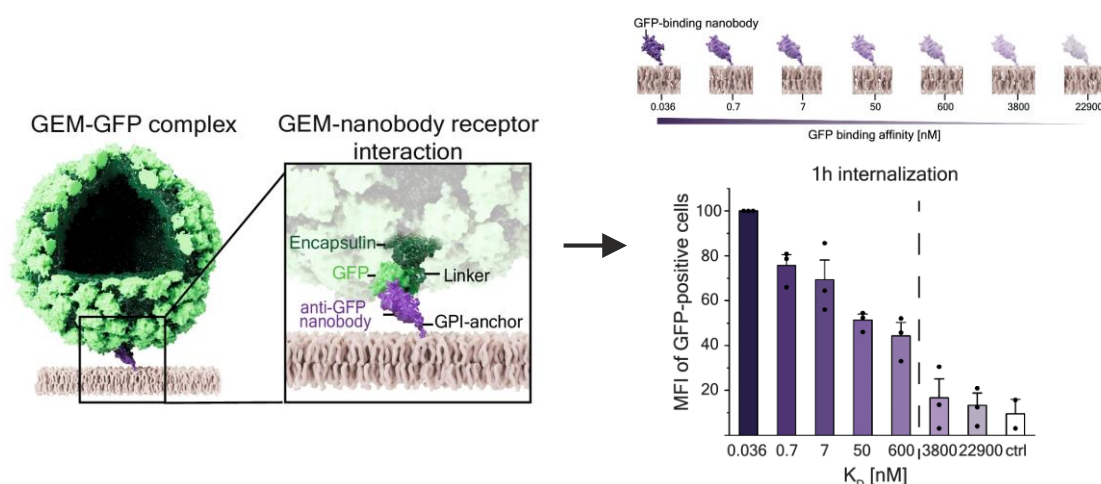


Figure 4 An adhesion energy threshold needs to be surpassed to induce nanoparticle uptake into target cells. Reprinted and adapted from Groza et al.^[70] Published Open Access, Springer Nature Limited. The article is licensed under a Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>). Figure 2A (<https://www.nature.com/articles/s41467-024-47109-7/figures/2>) and Figure 4A and 4D (<https://www.nature.com/articles/s41467-024-47109-7/figures/4>) were used and rearranged. Schematic representation of the polyvalent virus-like particle (nanocarriers) functionalized with GFP, and the binding structure (anti-GFP nanobody) inserted into the cell membrane. GFP-binding nanobodies with decreasing GFP binding affinity were incorporated in the plasma membrane of cells to proof that the internalization of nanocarriers follows a “yes” or “no” pattern depending on the binding strength. The endocytosis of the nanocarriers was determined from the mean fluorescence associated with these cells after 1 hour incubation. With decreasing GFP-binding affinity, both the amount of nanocarriers endocytosed into cells as well as the number of cells internalizing nanocarriers, dropped. The striking decrease of the samples with the lowest binding affinity receptors indicate a threshold value that needs to be exceeded for successful internalization of the nanocarriers.

When considering the overall picture, it is important to note that the three described parameters affecting the binding selectivity of nanoparticles are interrelated and their effects are closely intertwined.^[17,50] Jeppesen et al.^[54] concluded that the binding energy of a receptor-ligand pair determines the degree of tether flexibility required for the ligand to successfully scan the cell surface. The overall range, rate, and ultimate strength of complementary bond formation is thus dependent on both, the specific ligand-receptor pair interaction and the dynamics of the tethering chain. Consequently, all three factors need to be optimized in close coordination with each other to gain the most advantageous “keys” for selective binding.

1.4 Available Receptor Number

Shifting the perspective to the receptor-expressing target cells, the number of available receptors, “locks”, remains another important factor influencing the success of the interaction with the nanoparticles and the selectivity of the binding.^[17,50,68,74] Tan et al.^[68] developed a multivalent, multi-receptor, multi-ligand binding model to analyze mechanisms for cell-selective binding. Their model showed that the ratio of multivalent binding to cells expressing the same receptor at high level (target cells) and medium level (off-target cells) is maximized for systems carrying low-affinity ligands at

higher valence, compared to systems carrying high affinity ligands at lower valence.^[68] They entitled this the “high-valency, low-affinity selectivity effect”. To analyze the underlying mechanisms, they simulated the binding behavior of octavalent ligands on cells expressing either 10^3 or 10^4 receptors and plotted the distribution of binding valence relative to its frequency as a function of the affinity of the ligands (K_D value). The data analysis showed that cells with higher receptor density formed a greater amount of high-valence bonds even with low-affinity ligands, compared to cells with lower receptor density. The authors attributed this to the fact that cells with higher receptor density can compensate for the lower affinity of the ligands through denser spatial receptor abundance. As a result, the rate of secondary binding events between the multivalent ligands and the receptors on the cell surface is higher than the dissociation rate of the ligands from the receptors after initial binding.^[68] This aligns well with what has already been described for the nanoparticle design.

To summarize, the required ligand valence on the nanoparticle surface and the receptor density expressed by the target cells are two tightly intertwined factors that influence the selectivity of the interaction. A sharp and distinct differentiation between target and off-target cells, respectively superselectivity, is therefore only feasible within a very narrow range.^[75] In other words, nanocarriers, which are functionalized with a defined number of ligands (with a given receptor activity), exhibit superselective interactions only with cells with a narrow receptor density. However, the receptor density on cells is highly variable and may change depending on external and internal stimuli. Consequently, superselectivity can only be achieved with highly variable systems with tunable ligand and receptor densities and adjustable interaction strength.^[64]

2. Specificity in the Lock-and-Key Model

The previously mentioned influencing factors on selectivity all rely on the specific binding between the ligands on the nanoparticle surface and the target cell receptors, as described by the lock-and-key model. In classical binding studies, however, it is generally presumed that a fraction of the ligands non-specifically interacts with receptors and the surface in which they are embedded (Figure 5).^[28,76,77] Optimizing ligands so that they differ significantly in their binding affinity to target and off-target receptors poses a particular challenge, especially for multivalent systems. The multivalent nature of the binding leads to an enhancement in binding strength,^[21] resulting in even weak interactions with off-target receptors collectively contributing to strong binding (Figure 5). This may result in non-specific adsorption of nanoparticles to cells, which do not express the target receptors.^[47] At this point, the concept of a tailored lock-and-key interaction is overridden by the multivalent nature of the nanoparticle-cell binding. Angioletti-Uberti^[47] used a statistical mechanical model to quantify the effect of interactions between ligand-functionalized nanoparticles and off-target receptors. By varying either the number of targeting ligands or their binding strength to untargeted receptors, he illustrated that with the increase of one or both of these two variables the effect of non-specific nanoparticle binding becomes more pronounced. Above a certain threshold number of untargeted receptors, adsorption of nanoparticles to surfaces occurs even in complete absence of target receptors. Thus, the potential of ligands to interact non-specifically with others than the target

receptors represents a significant factor influencing the selectivity of the nanoparticle-cell interaction.^[47]

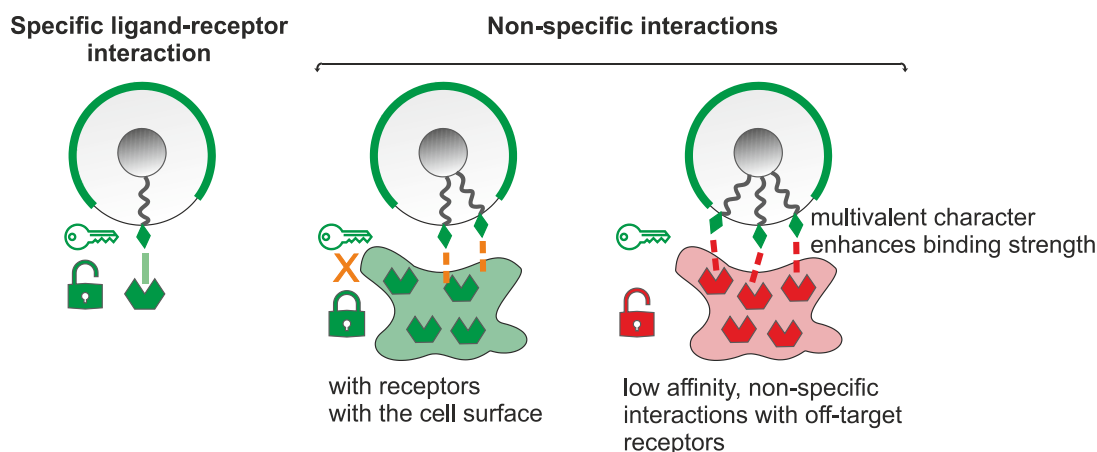


Figure 5 Non-specific interactions between ligand-functionalized nanoparticles and cells. Schematic representation of a specific ligand-receptor interaction, non-specific interactions of the ligands with the cell surface and receptors and low affinity interactions with off-target receptors. In the latter case, the multivalent character of the interaction can enhance the binding strength and thus promote the interaction between nanoparticles and off-target cells.

2.1 Surface Energy Driven Non-Specific Interactions

In the literature, however, non-specific nanoparticle interactions are primarily attributed to the nanoparticles' high surface energy.^[78] Non-specific interactions are thus discussed in the context of protein adsorption on their surface,^[79–82] the subsequent clearance of nanoparticles from the systemic circulation by the MPS,^[83–85] their charge-dependent^[86–88] and hydrophobic interactions,^[89–91] and their non-specific uptake in off-target cells and tissues when recognized by the organism as foreign material.^[92,93] Mitigating these interrelated effects increases the overall specificity of the nanoparticle-cell interactions. Thus, the focus shifts away from the potential of individual ligands to engage in non-specific interactions and towards the targeting efficiency of the entire multivalent nanoparticle. While these non-specific interactions are not captured in the traditional lock-and-key model, as they are not related to the failure of individual ligand-receptor interactions, they are, however, not independent of the ligand type and the ligand valence on the nanoparticles surface. As previously described, the ligand valence can influence the clearance of nanoparticles from the systemic circulation and affect their target/off-target ratio. Furthermore, the adsorption of proteins, beside to other factors (e.g. size, shape, and composition of the nanoparticles), also relies on their surface functional groups.^[81,94,95] Studies have demonstrated that the extent of protein corona formation is dependent on the hydrophobicity of the ligands and their conformation.^[96,97] Highly hydrophobic ligands, and their orientation relative to the nanoparticle scaffold, alter the protein corona around the particles.^[96] Zhang et al.^[98] found evidence suggesting that the molecular weight, and thus the bulkiness of ligands, might influence the composition and formation of the protein

corona. Their study employed sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and liquid chromatography-mass spectrometry to analyze the composition of the protein corona of polystyrene nanoparticles, which were functionalized with ligands exhibiting a range of molecular weights to target the transferrin receptor. The targeting efficiency of the differently functionalized nanoparticles to receptor-positive cells furthermore varied significantly, depending on whether they were previously incubated in serum-containing cell culture medium (assumed to correspond to an “in vitro corona”) or in human plasma (assumed to correspond to an “in vivo corona”) (Figure 6).

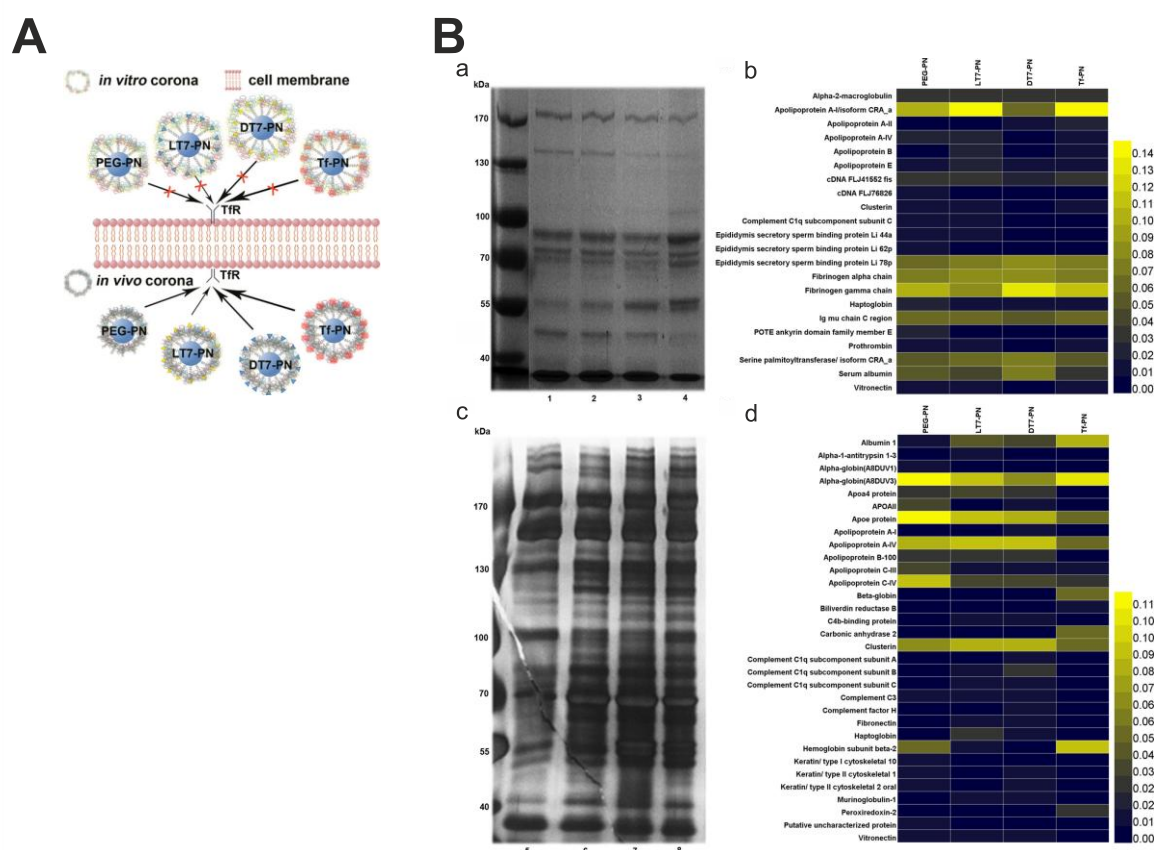


Figure 6 Ligand size and conformation affect the protein corona formation around the nanoparticles and the subsequent nanoparticle-cell interaction. (A)/(B) Reprinted (adapted) with permission from Zhang et al.^[98] Copyright (2025) American Chemical Society. (A) Schematic illustration of nanoparticles functionalized with transferrin receptor-targeting ligands of different molecular weight and the effects of in vitro and in vivo corona formation on their interaction with the receptor. (B) Analysis of the protein corona around the nanoparticles after incubation in serum-containing cell culture medium (“in vitro corona”) (a/ c) and in human plasma (“in vivo corona”) (b/ d). Polystyrene nanoparticles were functionalized with peptides of different conformation, LT7 (CHAIYPRH, $M_w < 1$ kDa) (LT7-PN) or DT7 (hrpyiahc, all D-form amino acids, $M_w < 1$ kDa) (DT7-PN), or transferrin, a large size protein ($M_w = 77$ kDa) (Tf-PN). PEGylated polystyrene nanoparticles (PEG-PN) were used as control. Coomassie blue stained SDS-PAGE gel of in vitro corona (a) and silver stained SDS-PAGE gel of in vivo corona (c) with lanes 1-4 indicate PEG-PN, LT7-PN, DT7-PN, and Tf-PN (in order). Nano-LC-ESI-MS/MS analysis of abundant proteins ($> 1\%$) represented as heat maps for in vitro (b) and in vivo (d) conditions.

The protein corona forming around the nanoparticles alters their synthetic identity and creates a new biological identity.^[78,81,99] On the one hand, it can affect the visibility and availability of ligands on the

nanoparticle surface for interactions with target receptors.^[98,100,101] On the other hand, the biomolecules present in the protein corona can mediate specific yet unintentional interactions with cell membrane receptors.^[80,102,103] Therefore, they determine the biological fate of the nanoparticles and can undermine their designed functions,^[104] as extensively reviewed elsewhere.^[94,105,106] To achieve non-specific binding resistance and to decrease the protein adsorption, the surface energy of nanoparticles needs to be lowered.^[107] Widely used approaches are the coating of nanoparticles with PEG^[108,109] or zwitterionic polymers.^[110,111] The antifouling capacity of these polymer coats relies on the formation a stable hydration layer around the nanoparticles either based on hydrogen bonding (PEG) or ionic salvation (zwitterionic polymers).^[111,112] The achieved “stealth” effect depends on the density and conformation of the spacer chains.^[113,114] Consequently, a compromise needs to be reached between the ligand density and the density of shielding spacer chains. This makes the ligand density not only a crucial factor for binding selectivity but also for binding specificity.

2.2 Contributions of Non-Specific Interactions to the Binding Behavior

An important aspect that should not be ignored when examining the relationships of specific and non-specific interactions is that the initial contact between nanoparticles and cells is frequently driven by the high surface energy of nanoparticles, and thus non-specific.^[78] Non-specific interactions can therefore significantly contribute to the adhesion force of the nanoparticles to the cell surface.^[115] This is also evident in a study conducted by Lundgren et al..^[11] They utilized quartz crystal microbalance with dissipation monitoring and waveguide light-scattering microscopy to investigate the interactions between differently sized biotin-functionalized/PEG-shielded gold nanoparticles and lipid bilayers with mobile streptavidin receptors (Figure 7).

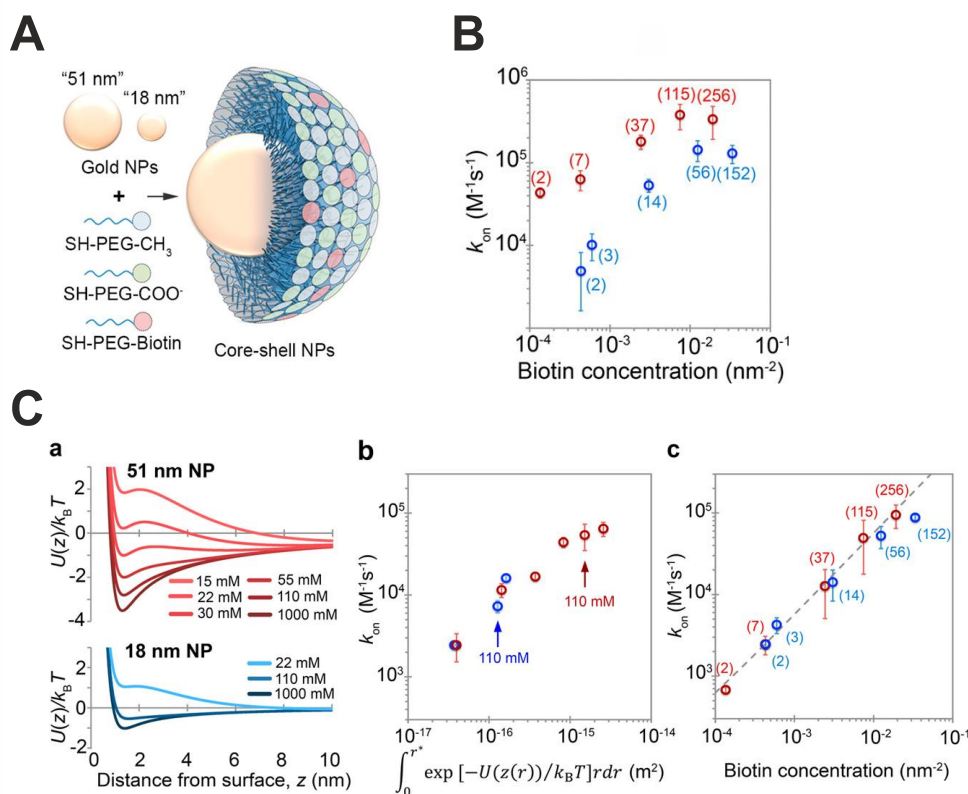


Figure 7 Evaluation of the impact of ligand density and non-specific interactions (DLVO type) on the interaction between differently sized (18 nm and 51 nm) biotin-functionalized/PEG-shielded nanoparticles and receptor-positive bilayers. Reprinted adapted with permission from Lundgren et al..^[11] Copyright (2025) American Chemical Society. (A) Schematic illustration of the model nanoparticle system. (B) Binding rate constants, k_{on} , for 18 nm-sized (blue) and 51 nm-sized nanoparticles (red) as a function of the biotin ligand concentration on their surface (nm^{-2}), with the average number of biotin molecules per nanoparticle indicated within parentheses. (C) The influence of the nanoparticle-membrane interaction potential on their binding rate constants, k_{on} (18 nm-sized nanoparticles indicated in blue and 51 nm-sized nanoparticles indicated in red). (a) Depiction of the correlation of the interaction potential ($U(z)/k_B T$) and the distance between the periphery of the PEG brush of the nanoparticles and the lipid bilayer for different ionic strengths (in mM). (b) Binding rate constants, k_{on} , for 18 nm- and 51 nm-sized nanoparticles with similar ligand coverage as a function of the different interaction potentials depicted in (a). (c) Binding rate constants, k_{on} , for 18 nm- and 51 nm-sized nanoparticles as a function of the biotin ligand concentration (nm^{-2}) at similar binding conditions with respect to their interaction potential to the lipid membrane. The k_{on} values converge to form a linear function. This indicates that the size-dependent differences in the binding rates under physiological conditions (B) are attributed to size-dependent colloidal interactions.

Their results indicate that only in the complete absence of non-specific interactions, the binding rate of targeted nanoparticles is solely dependent on their ligand valence. By correlating the binding rates (k_{on}) of ligand-functionalized nanoparticles with those of non-functionalized nanoparticles, they were able to demonstrate the influence of size-dependent weak colloidal interactions (DLVO type) between the nanoparticles and the lipid bilayers. These colloidal interactions lead to a heterogeneous size-dependent distribution of the nanoparticles relative to the membrane and influence their association rate. They demonstrated that even a slightly attractive van der Waals potential in the interaction between the nanoparticles and the membrane led to a significant increase in the binding rate. These attractive forces prolong the residence time of nanoparticles in close proximity to the membrane and thus increase the probability for specific ligand-receptor interactions. The colloidal interactions exhibit

a positive correlation with the nanoparticle size. Consequently, larger nanoparticles can display a higher avidity towards the receptor carrying membrane compared to smaller particles of the same type, even when the number of their ligand-receptor interactions is the same. This clearly evidences the influence of non-specific interactions on specific binding between ligand-functionalized nanoparticles and receptor carrying surfaces. Furthermore, also transport and uptake processes of nanoparticles in cells are affected by a combination of specific and non-specific interactions.^[116,117]

3. The Accessibility of the “Keys” on the Nanoparticle Surface

The distribution of ligands on the surface of nanoparticles is mostly assumed to be uniform, and studies regularly refer to an average ligand number per particle.^[118] However, when discussing the availability of ligands, it is essential to critically assess whether the ligands are indeed homogeneously distributed and whether the calculated or intended ligand density on the nanoparticle surface corresponds to the actual number of accessible ligands. As previously described, an adequate availability of ligands on the surface of nanocarriers is crucial for the establishment of specific and selective interactions. Several factors that may affect the accessibility or “visibility” of ligands, such as steric effects and the formation of a protein corona, have already been discussed. Additionally, inter- and intra-particle heterogeneities that may arise during the production and functionalization process need to be considered. These can affect the structure of both, the nanoparticle core and their ligand-functionalized surface. Dao et al.^[119] demonstrated in a study on hybrid polymer/lipid particles that these tend to phase-separation, leading to the formation of nanodomains on their surface enriched in either polymers or lipids. The cover density of polymer coats, such as PEG, can also be heterogeneous, which appears as variations in the corona thickness or uncovered areas on the surface of nanoparticles.^[118] These structural heterogeneities have been summarized in read-worthy reviews and viewpoints by Rabanel et al.,^[118] Stavis et al.,^[120] and Chen.^[121] Interested readers are referred to their work for further detail. At this point, however, a closer look should be taken at the heterogeneities in ligand number and distribution on the nanoparticle surface.

The functionalization of nanoparticles with ligands can be achieved in two ways: either by the self-assembly of functionalized substrates (e.g. polymers or lipids) as building blocks, or by post-grafting reactions of pre-existing nanoparticle cores (e.g. gold or silica nanoparticles).^[12,56] In the latter case, different conjugation methods can lead to variations in the coupling efficiency.^[122] Contributing to this is the fact that not all coupling sites on the surface of nanoparticles are necessarily available.^[123,124] Nirmalananthan-Budau et al.^[124] demonstrated in a study on polymer and silica nanoparticles bearing amino or carboxy groups that not all of these coupling sites were effectively available. Consequently, the nanoparticles could only be functionalized with ligands to a certain percentage. The described synthetic methods, based on either an arbitrary assembly of building blocks or random collision and attachment events between ligands and nanoparticles, thus promote heterogeneous ligand-nanoparticle distributions, which are frequently underestimated.^[125] This also affects the nanoparticles’ binding behavior and kinetics towards targeted surfaces.^[61] Li et al.^[61] revealed that

batches of nanoparticles, which display heterogeneous ligand distribution, show discrete binding activities within their subpopulations and thus differ in their association and dissociation kinetics on surfaces. Controlling the homogeneity of nanoparticles is a technically and analytically complex endeavor.^[120] Delcanale et al.^[126] introduced with DNA-PAINT an innovative technology for visualizing the nanoscale topography on a single particle level (Figure 8A). Employing this nanoscopy technique, they were able to resolve the spatial distribution and functional heterogeneity of one or more ligands on the surface of nanoparticles. To localize single molecules, the transient interaction between two complementary DNA strands was utilized, one of which was linked to a fluorescent probe. In this way, the authors were able to visualize cluster formation in the distribution of surface functionalities (Figure 8B). They attributed this among other factors, to steric hindrance between the ligands. Other studies have shown that chemical microenvironments can form around ligands, e.g. arising from shielding effects between the ligands or different ion concentration and polarity gradients.^[127,128] As a result, ligands can mutually influence each other in their symmetry and orientation. This leads to heterogeneity in their symmetrical alignment on the particle surface and variations in their dynamic behavior.^[127]

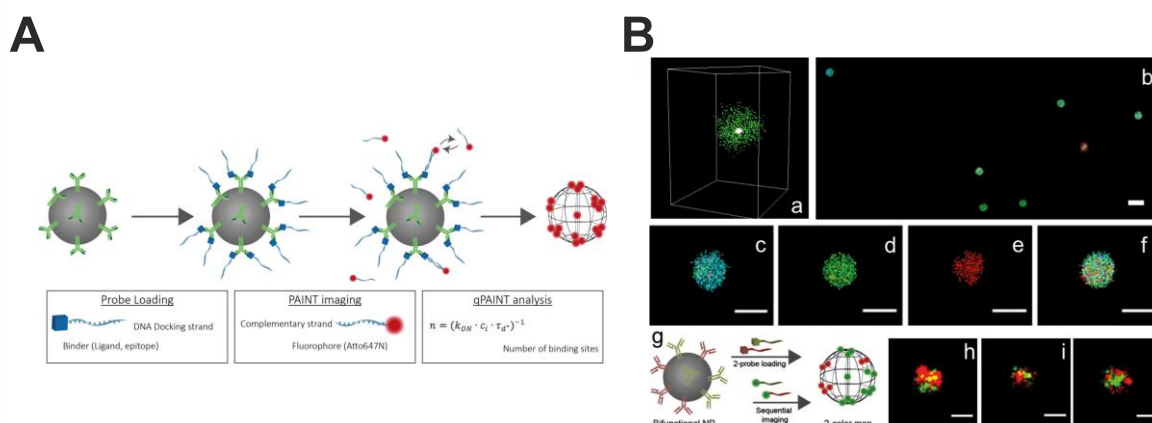


Figure 8 Cluster formation and shielding effects between ligands. (A)/(B) Reprinted with permission from Delcanale et al.^[126] Copyright (2025) American Chemical Society. (A) Schematic illustration of the DNA-PAINT technology. (B) 3D DNA-PAINT images of nanoparticles functionalized with either one ligand type or multiple ligand types (multicolor images). (h-j) The multicolor images show the heterogeneous distribution of the ligands on the nanoparticle surface and indicate their cluster formation.

The limited availability of ligands on the nanoparticle surface, along with inter- and intra-nanoparticle heterogeneities in their distribution and the formation of microdomains, could be related to non-linear effects observed during the optimization of nanoparticles.^[118] Furthermore, the innate heterogeneity of the surface topography can lead to an acquired heterogeneity due to different protein attachments after nanoparticle application, thus influencing their *in vivo* fate.^[118] Taken together, this creates a picture that does not align well with the idealized availability of ligands as “keys” for interactions with target receptors according to the lock-and-key model (Figure 9A).

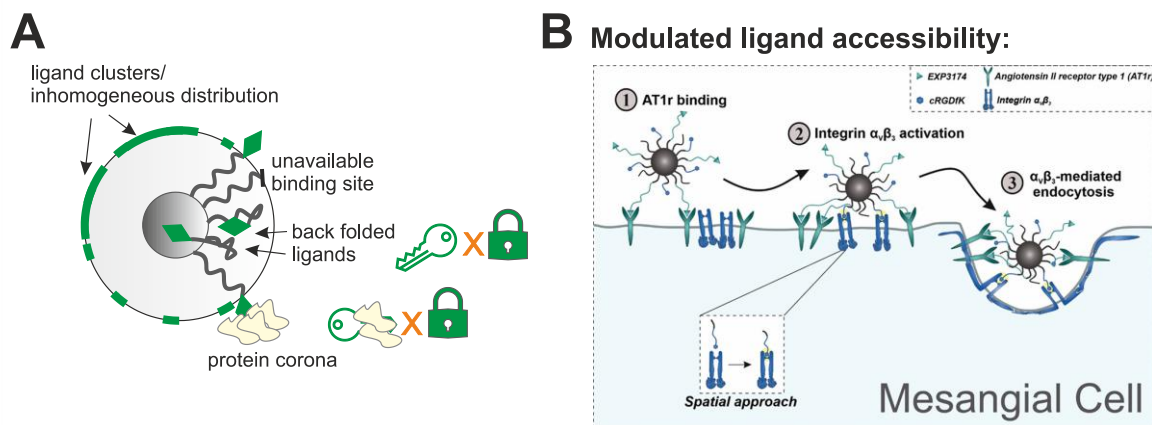


Figure 9 Confined accessibility of ligands “keys” on the nanoparticle surface. (A) Schematic representation of reasons for the limited accessibility of ligands for interactions with the target receptors. (B) Reprinted with permission from Fleischmann et al.^[41] Copyright (2025) American Chemical Society. Schematic illustration of the sterically controlled, sequential nanoparticle-cell interaction. The polymer nanoparticles were functionalized with two types of ligands coupled to polymer chains of different lengths. The nanoparticle initially bound to the cell surface via interactions between the ligand EXP3174, which was coupled to PEG5k polymer chains, and its target receptor. Subsequently, integrin-mediated endocytosis was triggered through interaction between cRGDFK, the second ligand, which was coupled to shorter PEG2k chains, and the integrin receptors on the cell surface.

An alternative perspective on this topic is to utilize the limited availability of ligands^[129] or an engineered heterogeneity in their functionalization to optimize the biomedical performance and cell specificity of the nanocarriers.^[130,131] This encompasses on the one hand chemically anisotropic nanoparticles and superstructures like Janus-particles, multicompartiment and patchy particles.^[118] These interesting design concepts have been reviewed elsewhere.^[132,133] And it includes on the other hand design approaches, which consciously use the masking of ligands by surrounding polymer chains. For instance, the coupling of ligands to linkers of varying chain length results in their sequential presentation to the target cells (Figure 9B).^[41,134] The ligands, which are attached to the shorter chains, are initially concealed for interactions with target receptors, and then exposed after successful binding of the ones coupled to longer linker chains. This two-step recognition contributes to an increased cell specificity of the binding. Alternatively, ligands can be associated with cleavable lipophilic protective groups, which promote the folding of the ligands into the more lipophilic nanoparticle core.^[135] After stimuli-responsive cleavage of the protective groups, the ligands “pop-up” and unfold together with their linker chains due to their increased hydrophilicity. The hydrophilicity of the ligands is consequently another crucial factor that determines the orientation of the ligands relative to the nanoparticle scaffold, thereby influencing the binding behavior to the target receptors.^[59]

4. An Extended View on the Lock-and-Key Model

For the functionality of the cell membrane, it is crucial that it possesses spatio-temporal structuring to delineate various functional regions from one another.^[136,137] Consequently, the cell membrane is

laterally heterogeneous with nano-scale lipid rafts and protein clusters.^[138] These can alter their localization and areal expansion in response to activation events, such as the binding of multivalent ligands.^[137] Proteins within the same lipid phase can sense attractive forces, enabling them to approach each other.^[138] Furthermore, studies of the cell membrane have shown that it exhibits hotspots for certain receptor types, which are partially defined by the cytoskeleton and clathrin-coated pits (CCPs).^[139] Cell membranes restructure themselves via various mechanisms. For instance, clathrin-mediated endocytosis is an important process for the uptake of receptors and transporters in eukaryotic cells and thus essentially contributes to the remodeling and structuring of the cell membrane.^[140,141] Depending on the receptor type, the occurrence of CME proceeds either continuously and irresponsibly of ligand occupancy, or conversely only after binding of a ligand to the receptor.^[140] In the latter case, activation by a ligand leads to the recruitment of adaptor proteins on the cytoplasmic side of the cell membrane, targeting the receptors to CCPs.^[142,143] In the region of CCPs, the membrane is deformed towards the cytoplasm stabilized by the clathrin scaffold.^[142] CCPs can occur at various locations within the plasma membrane, leading to a local condensation of receptors in pit-shaped structures.^[141] Besides for endocytic processes, it is known that many target receptors are clustered in microdomains on the cell membrane to facilitate signal amplification and robustness of cellular signaling.^[144–147] Viruses have undergone evolutionary optimization to form specific patterns with their binding entities, e.g. by temporarily clustering the spikes on their surface and thus matching best the receptor structuring and geometry or to form condensed interactions with receptor microdomains.^[36] In a recent study on the reovirus, the impact of its capsid protein architecture and arrangement in receptor interactions was investigated utilizing atomic force microscopy in combination with molecular dynamics simulations (Figure 10A).^[148] The capsid of the reovirus is structured in heterohexamers. Receptors are bound at the interface between two adjacent heterohexamers. The geometry of the virus particles plays a decisive role in the interaction with the curved binding interfaces of the receptor, one of which is convex and one concave (Figure 10B). This reveals optimization potential regarding the geometric arrangement of ligands on the surface of nanocarriers.

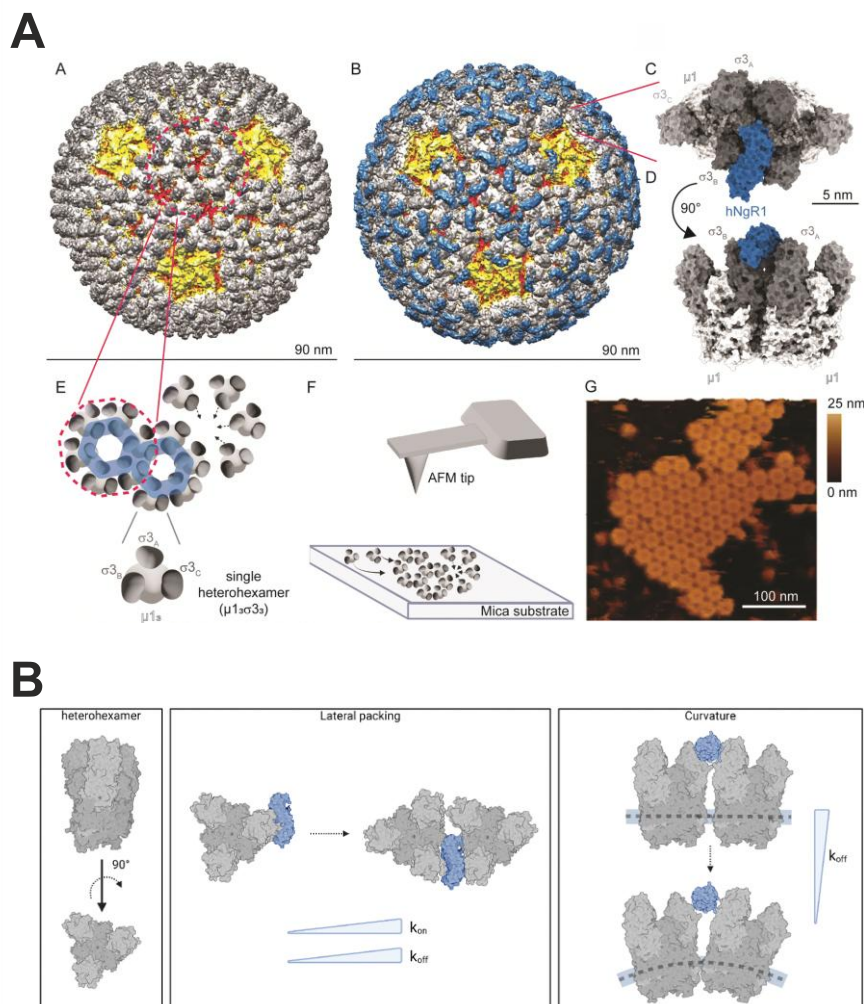


Figure 10 Impact of the capsid protein architecture and arrangement of reoviruses on their target receptor interaction. Reproduced and adapted from Dos Santos Natividade et al. (Ref.^[148]) with permission from the Royal Society of Chemistry. The article is licensed under a Creative Commons Attribution 3.0 International License (<https://creativecommons.org/licenses/by-nc/3.0/>). Figure 1 (https://pubs.rsc.org/image/article/2024/nh/d4nh00315b/d4nh00315b-f1_hi-res.gif) and Figure 5 (https://pubs.rsc.org/image/article/2024/nh/d4nh00315b/d4nh00315b-f5_hi-res.gif) were used in parts and rearranged. (A) Surface structuring of the capsid of the reovirus. (A) Density map of the reovirus capsid. (B) Density map of the reovirus capsid in interaction with the target receptor. (C)/ (D) Two heterohexamers on the virus surface (depicted in gray) in complex with the target receptor, hNgR1 (depicted in blue). (E) Schematic assembly of the heterohexamers in hexagonal structures. (F) Imaging set up for AFM measurements. (G) AFM topography of a mica substrate functionalized with hexagonal targeting structures of the viral capsid. (B) Schematic illustration of the spatial organization and viral curvature as critical determinants for the reovirus-receptor complex with the receptor being depicted in blue and the viral structures in gray.

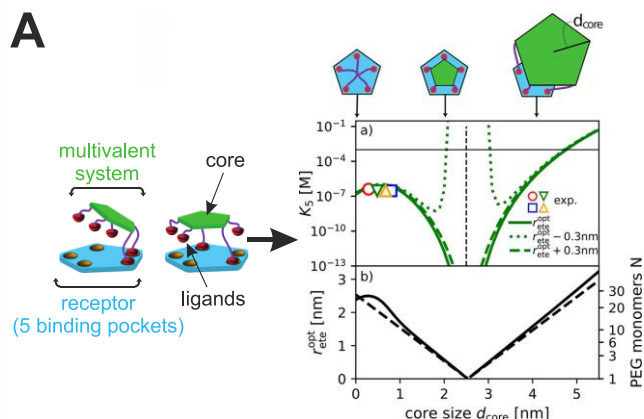
4.1 Geometric Binding Patterns as the First Dimension of Superordinate Lock-and-Key Motifs

When developing multivalent systems, their geometric optimization can be approached in different ways. From a thermodynamic perspective, the binding affinity is enhanced by adjusting the size of ligand patches and the distance between the individual ligands to match binding pockets on multivalent receptors, or respectively receptor patterns on the cell membrane.^[50,149] Otherwise, the ligands could be sterically excluded from binding by adjacent ligand-receptor bonds.^[150] The

previously described study by Liese and Netz^[50] assumed a model receptor with five binding pockets arranged in a regular polygon (Figure 11A). The binding partner was depicted as a multivalent system consisting of a core functionalized with five ligands coupled to linker chains. They found the highest binding affinity to be achieved, when the core size of the multivalent system matched the receptor size. Moreover, the length of the linkers contributed to the affinity gain when it was slightly longer than the distance between the core of the multivalent system and the receptor binding sites. The authors concluded that both, core size and linker length, play a decisive role in the geometric optimization of multivalent systems towards multivalent receptors. Furthermore, they hypothesized that geometric matching enables multivalent systems to discriminate between receptors of different sizes. This is a revolutionary idea as it contributes to a broader understanding of target selectivity.

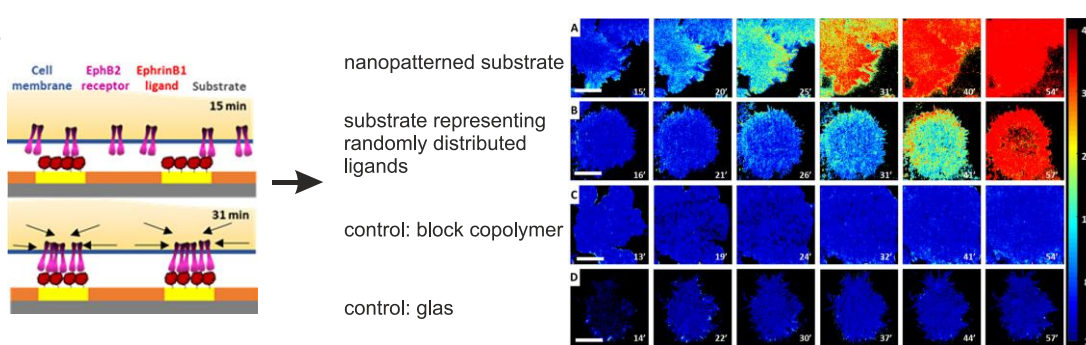
Geometric optimization

A



Receptor clustering

B



Geometric congruence

C

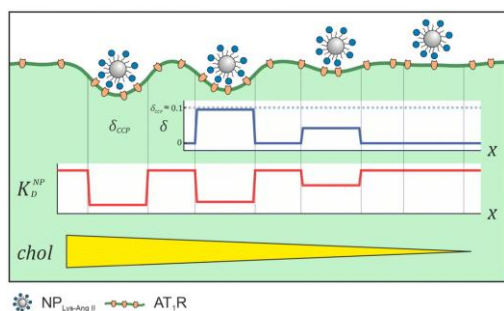


Figure 11 Geometric binding patterns and congruence in the interactions between nanoparticles and target receptors and cells. (A) Reprinted adapted with permission from Liese and Netz.^[50] Copyright (2025) American Chemical Society. (a) Influence of the core size (d_{core} [nm]) of the pentavalent system on its dissociation constant (K_5 [M]) from the receptor. Model predictions for the optimal linker length r_{ete}^{opt} (solid line), for a linker that is shorter ($r_{ete} = r_{ete}^{opt} - 0.3$ nm; dotted line) and a linker that is longer ($r_{ete} = r_{ete}^{opt} + 0.3$ nm; dashed line) than optimal are shown. The dissociation constant of the multivalent system is the smallest, if the size of the ligand core is similar to that of the receptor (denoted by a vertical broken line). (b) Relationship between the optimal linker length (r_{ete}^{opt} [nm]) and the core size (d_{core} [nm]) of the multivalent system as solid line. The optimal linker length is slightly larger than the difference between core size and receptor size, which is depicted as broken line. (B) Reprinted adapted with permission from Hortigüela et al.^[151] Copyright (2025) American Chemical Society. Schematic depiction of the surface-bound ephrin B1 ligands and the membrane-bound EphB2 target receptors and oligomerization maps of the receptors at several timepoints after stimulation with ligands presented in nanopatterns or randomly distributed. The controls showed no receptor clustering. (C) Reproduced from Zimmer et al. (Ref. ^[152]) with permission from the Royal Society of Chemistry. The article is licensed under a Creative Commons Attribution 3.0 International License (<https://creativecommons.org/licenses/by-nc/3.0/>). Figure 4D was reprinted

(https://pubs.rsc.org/image/article/2023/nh/d2nh00543c/d2nh00543c-f4_hi-res.gif). Schematic illustration of the relationship between the membrane wrapping fraction δ and the nanoparticle avidity K_D^{NP} .

Another approach for geometric optimization was presented by Poon et al.,^[153] who engineered folate microclusters on the surface of micelles. By measuring the cell associated fluorescence of receptor-positive target cells after incubation with the functionalized nanocarriers, they proved microscale patterning of ligands to be a promising concept for optimizing multivalent kinetics and targeting efficiency. The superiority of surface clustering over a dispersed distribution of ligands for the internalization of nanoparticles was furthermore confirmed in a study on polystyrene nanoparticles.^[73] However, a main challenge in this context is, as described in the previous, the controlled and reproducible functionalization of the nanoparticle surface.^[86] In their in-depth review, Hu et al.^[154] discussed recent advancements in nanofabrication methods to design surface patterns on nanoparticles in the context of their biorecognition. The interested reader is pointed to their work for further information on this topic.

Switching the perspective to the receptor-expressing target cells, it has been shown that nano-patterned multivalent surfaces can also induce changes in receptor distribution, e.g. promoting receptor oligomerization or clustering on the cell membrane.^[15,151] Hortigüela et al.^[151] employed the multivalent interaction between surface-bound ephrin B1 ligands and membrane-bound EphB2 target receptors to demonstrate, that the spatial arrangement of ligands in patterns affects the clustering efficiency and kinetics of the receptor (Figure 11B). In live-cell experiments on HEK293T cells, they furthermore showed that the same ligand presented as spatial arranged nanopatterns, compared to randomly distributed ones, led to faster and stronger oligomerization of the target receptors. This reduced the ligand valence required to trigger the same reaction at the receptor by a factor of nine. The authors concluded that the presentation mode of ligands in multivalent systems can manipulate the receptor aggregation behavior on cell membranes. Thinking of ligand-functionalized nanocarriers, the spatial arrangement of their ligands can thus influence the structuring of the surface of host cells. Increasing receptor density in the contact area between the multivalent system and the cell membrane leads to a decrease of the receptor number in the vicinity. Therefore, the diffusion kinetics of the receptors can influence the ligand-receptor interactions of the multivalent system.^[155] Since the redistribution of receptors also affects membrane regions not directly involved in the binding of the multivalent system, binding should thus be considered as a global event rather than a local event.^[115] Nevertheless, membrane-bound receptors are often regulated in their conformational fluctuations by membrane confinements, depending on their signaling pathways.^[156]

Another perspective on matching structures is to consider them in the context of the geometric congruence between the cell membrane morphology and the nanoparticles.^[26,157] Defining nanoparticles as spherical structures with a diameter smaller than 100 nm,^[158] their geometry is matched perfectly on the cell membrane by dome-shaped CCPs.^[159,160] In these regions the membrane is already curved towards the particle and the bending energy required to overcome is thus lower.^[152,161] Additionally, the larger contact area between the nanoparticles and the cell

membrane statistically increases the number of binding events compared to the flat membrane.^[26,152] CCPs furthermore play a decisive role in the receptor-mediated endocytosis of viruses and nanoparticles.^[49,162] Our group has recently demonstrated in a both experimental and theoretical study that the avidity of ligand-functionalized nanoparticles on target cells is associated with the presence of CCPs (Figure 11C).^[152] We confirmed this correlation by treating the cells with inhibitors for CCP formation, thereby flattening the cell surface. The induced changes in the membrane morphology resulted in up to a 100-fold decrease of the nanoparticles' avidity. Our work thus revealed the geometry alignment between cell membrane morphology and ligand-functionalized nanoparticles to be a significant factor influencing nanoparticle-cell interactions. Our findings were supported by a study conducted by Li et al.^[49] The authors combined super-resolution photoactivation localization microscopy (PLAM) with single particle tracking to visualize the interaction between carboxylate-modified polystyrene nanoparticles of different sizes and CCPs. To make the CCPs visible for PALM, the employed cell line was transfected to express fluorescent clathrin chains. By spatially and temporally comparing the nanoparticle trajectories and the localization of the CCPs on the cell membrane, the authors were able to identify two pathways through which nanoparticles interact with CCPs. For unfunctionalized particles, the formation of CCPs occurred quantitatively (> 90 %) around them after they had encountered the cell surface. Analysis of the trajectories of these nanoparticles revealed that they did not diffuse freely across the membrane, but their movement was closely related to the newly forming CCPs. Only a small fraction of the unfunctionalized nanoparticles moved on the cell surface to pre-existing CCPs and were endocytosed by them. When transferrin was coupled as a ligand to their surface, a larger fraction of the nanoparticles diffused to the pre-existing CCPs. The ligand-functionalized nanoparticles exhibited a larger mobile fraction on the cell membrane compared to the unfunctionalized nanoparticles. The ligands mediated the specific interaction with pre-existing receptor clusters in CCPs, while the nanoparticles diffuse across the cell surface.

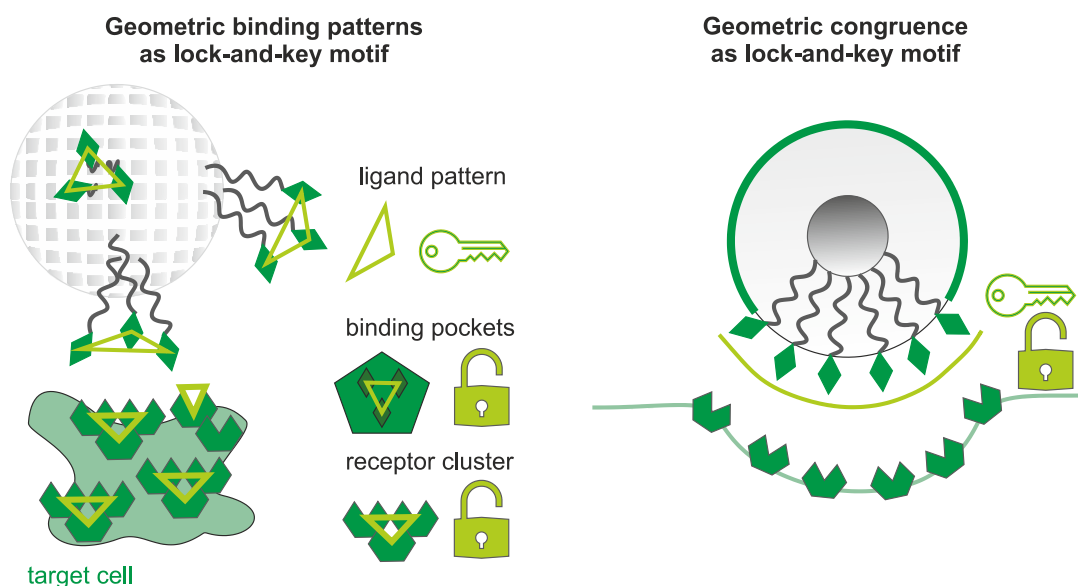


Figure 12 Schematic depiction of geometric binding patterns as superordinate lock-and-key motifs.

Based on the so far described findings, an expanded understanding of the lock-and-key principle can be derived: The entire recognition structure, e.g. the nanoparticle structure (core) or the ligand pattern can be interpreted as the “key” (Figure 12). It furthermore appears advisable to extend the understanding of the “lock” from individual receptors to either already existing or ligand-induced geometric receptor clusters.

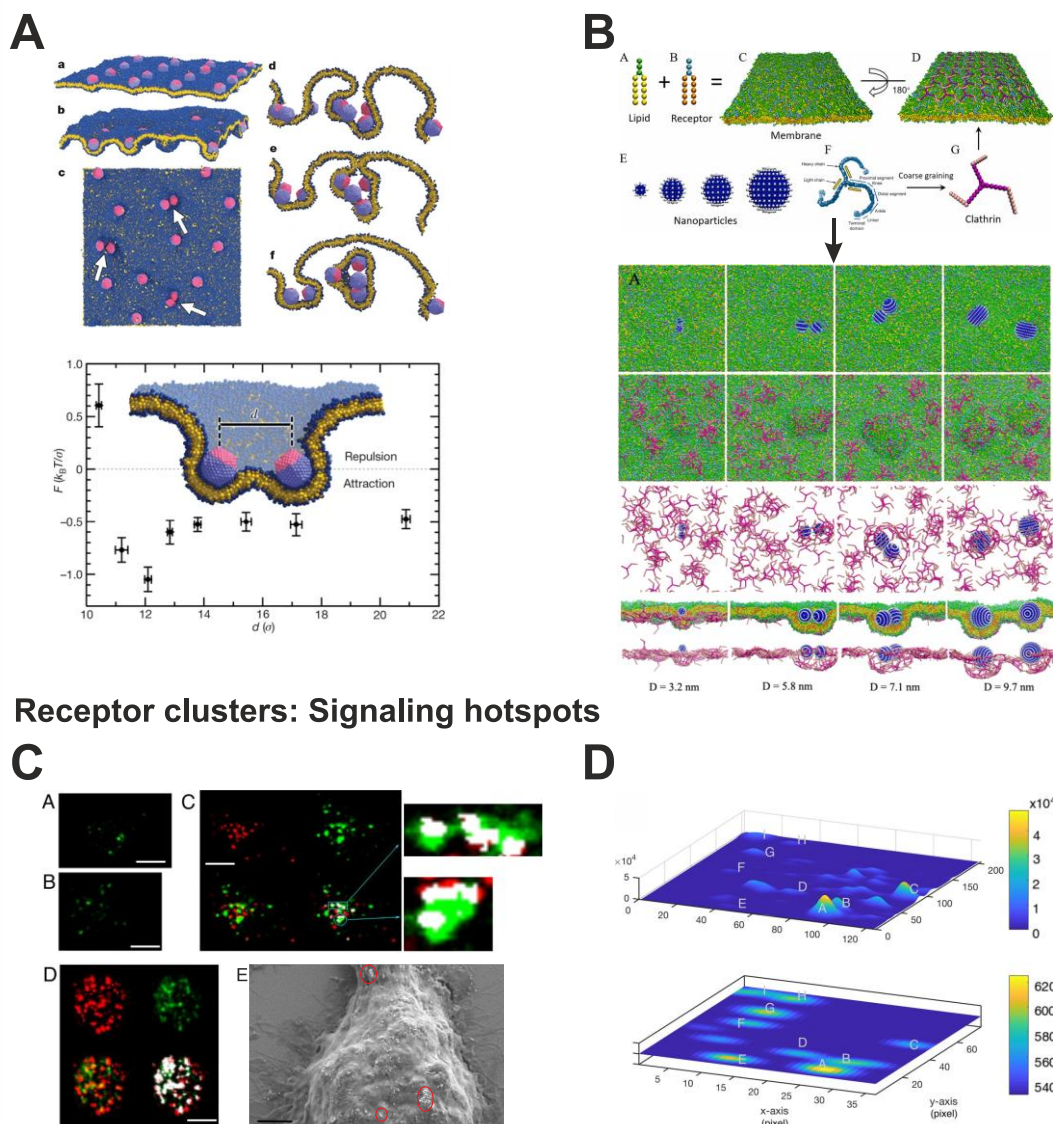
4.2 Cluster Formation as Second Dimension of Superordinate Lock-and-Key Motifs

Like receptors, nanoparticles can form clusters and, either during storage or after contact with biological media, aggregate into various shapes and sizes.^[86,163] This may affect how they are recognized by cells and, consequently, their interactions with the target structure. In their study on PEGylated gold nanoparticles, Lin et al.^[164] showed that the nanoparticles were extravasated from the blood vessel as clusters and, as such, taken up by macrophages and transported into the tumor tissue. Our group demonstrated by dynamic light scattering (DLS) measurements and transmission electron microscopy (TEM) images that the poly(lactide-co-glycolide)/poly(ethylene glycol)-b poly(lactide) (PLGA/PLA-PEG) core-shell nanoparticles under investigation exhibited different tendencies towards agglomeration. Their agglomeration behavior was dependent on the degree of their functionalization and the length of the utilized PEG-linkers (PEG2kDa, PEG3.5kDa, PEG5kDa).^[51] Nanoparticles, which were coated with short PEG-linkers (PEG2kDa), fully functionalized with cRGD as ligand, showed a strong tendency to form clusters. We calculated the absolute ligand valence of the formed nanoparticle clusters in relation to their surface. Correlating this with the cell-associated fluorescence of the nanoparticles on target receptor-positive cells, we were able to show that the nanoparticles exhibited cooperative binding within the cluster structures.

Alternatively, nanoparticles encounter the cells independently and can aggregate into clusters on the cell surface. The cluster formation and subsequent uptake mechanisms represent processes that are influenced by the applied nanoparticle concentration.^[165] On membranes, the aggregation of nanoparticles can occur entirely independently of the attractiveness of the particles surfaces to each other, based solely on membrane curvature-mediated interactions.^[166,167] Those interactions can be either attractive or repulsive.^[168] In coarse-grained membrane simulations, Reynwar et al.^[169] were able to show that colloidal spheres exert attractive forces on each other when a certain inter-particle distance is not exceeded (Figure 13A). The colloidal spheres thus tend to form pairs, to minimize the bending energy of the membrane. Similarly, receptor-mediated endocytosis, which occurs after successful ligand-receptor interactions, can proceed as a cooperative process involving multiple nanoparticles. Yue et al.^[170] simulated the endocytosis of ligand-functionalized nanoparticles on a receptor-carrying membrane using N-varied dissipative particle dynamics, a computer simulation technique. They demonstrated the receptor-mediated endocytosis of nanoparticles to be a cooperative process, depending on the membrane tension and the nanoparticle size and concentration. For small particles, clustering reduces the curvature energy of the membrane and, consequently, the stored elastic energy. Albeit, with increasing particle size, the nanoparticles appear to repel each other again. For larger particles, the total energy of their ligand-receptor interactions

exceeds the energy required to bend the membrane, while at the same time they compete for binding sites. The competition for a limited number of receptors, however, makes their interaction with the cell membrane interdependent and turns it into a global event.^[163] A pitfall of their model is, that Yue et al.^[170] simulated a “pure” bilayer and did not consider the effect of clathrin assembly underneath the membrane. Clathrin makes the endocytic process, as previously described, highly size selective. While the endocytosis of small nanoparticles is favored compared to the “pure” plasma membrane, a size mismatch occurs between larger nanoparticles and the formed clathrin cage.^[171] Another DPD simulation study displayed, that clathrin influences the cooperative endocytosis of nanoparticles (Figure 13B).^[171] It was demonstrated that clathrin accumulated in the wrapping region around the nanoparticles and affects the balance between adhesion energy, respective ligand-receptor-binding, and bending energy.^[171] This contributes to a more precise understanding of cooperative nanoparticle interactions on natural membranes. However, the fundamental tendencies, namely that smaller nanoparticles are more likely to be cooperatively endocytosed while larger nanoparticles interact with the membrane independently, were confirmed. The correlations become more intricate when considering polymer-grafted nanoparticles. As previously discussed, polymer brushes are intended to reduce non-specific interactions and contribute to stabilizing effects between the nanoparticles through steric repulsion (“polymeric stabilization”).^[172,173] The nanoparticle diameter, the chain length of the polymers, and the grafting density influence the attractive or repulsive forces acting between nanoparticles.^[173,174] The latter must be overcome for cooperative endocytosis of multiple nanoparticles through membrane curvature-mediated interactions.

Nanoparticle clustering on the cell membrane



Receptor clusters: Signaling hotspots

Figure 13 Cluster formation of nanoparticles and target receptors. (A) Reprinted adapted from Reynwar et al.^[169] with permission from Springer Nature (License Number: 6027230203998). Simulation of a fluid bilayer composed of three-bead lipids with one hydrophilic head-bead (indicated in blue) and two hydrophobic tail-beads (indicated in yellow). The capsids (indicated in purple and blue) represent nanoparticulate systems, whose surfaces exhibit no mutual attraction. A series of simulation snapshots of the cooperative budding driven by 16 capsids on the membrane is shown, as well as the correlation between force ($F(k_B T/\sigma)$) and distance ($d(\sigma)$) between two capsids on the membrane. (B) Reprinted adapted from Li et al.^[171] Copyright (2025) with permission from Elsevier (License Number: 6027230833287). Schematic representation of the model system and simulation of clathrin-mediated endocytosis for two nanoparticles of the same size. Data are shown for nanoparticles with diameters of 3.2 nm, 5.8 nm, 7.1 nm and 9.7 nm. (C)/(D) Reprinted adapted with permission from Zhang et al.^[146] Copyright (2025) American Chemical Society. (C) Confocal images and SEM image. (A) Immunolabeled target receptor (pEGFR) in the dorsal membrane of the target cell line after treatment with the free ligand. (B) Control experiment: immunolabeled target receptor after treatment with the supernatant derived from ligand-functionalized nanoparticles. (c) Top left: confocal backscattering image of the ligand-functionalized nanoparticles bound to the plasma membrane of the target cell line. Top right: confocal scan of the immunolabeled target receptors. Bottom left: merged channels. Bottom right: computed colocalized map between nanoparticle signal and receptor signal. (D) Same as in (c) with nanoparticles functionalized with a higher ligand density. (E) SEM image of ligand-functionalized nanoparticles on the target cell membrane. Nanoparticles formed clusters on the cell membrane. Several nanoparticle clusters are exemplarily highlighted in the image using red circles. (D) Top: Confocal map of the target receptor. Bottom: Localization of the ligand-

functionalized nanoparticles determined from the localized surface plasmon resonance (LSPR) wavelength of the nanoparticles. A positive correlation between the LSPR wavelength and the receptor signal was observed.

The formation of nanoparticle clusters can be closely intertwined with the presence or emergence of receptor clusters on the cell membrane. Zang and Reinhard^[146] demonstrated this connection in their study. They showed that the spatial clustering of ligand-functionalized nanoparticles represents an additional mechanism to locally increase the ligand-receptor interaction density on the cell membrane. This is particularly relevant for receptors that can aggregate into higher-order assemblies, such as the epidermal growth factor receptor (EGFR) (Figure 13C). The authors conducted their study using EGF-functionalized gold nanoparticles with known ligand valence. Since EGFRs are typically distributed on the cell membrane in clusters of an average size of 150 nm, the nanoparticles were optimized in their size for concurrent binding of multiple nanoparticles per cluster. In two EGFR-positive cell lines, they demonstrated a correlation between nanoparticle-mediated EGFR phosphorylation and the formation of nanoparticle clusters. The authors compared confocal fluorescence scans of fluorescently labeled target receptors on the cell surface with dark-field scattering spectra maps of the ligand-functionalized nanoparticles (Figure 13D). Through this approach, they were able to prove that the nanoparticle clustering on the cell surface induced the formation of phosphorylation “hot spots”. Therefore, receptor clustering and nanoparticle clustering are intercorrelated in this system: A locally increased receptor density due to receptor oligomerization contributes to the enhanced accumulation of EGF-functionalized nanoparticles in this region and thus to the formation of nanoparticle clusters. Conversely, the nanoparticle clustering amplifies the effect of EGFR phosphorylation, thereby contributing to signal amplification through lateral receptor activation.^[175] This, in turn leads to the di- and oligomerization of additional receptors.^[176] Meanwhile, efforts are also being made to intentionally assemble nanoparticles into colloidal systems to modulate their cellular interactions and binding strength.^[177] Ohta et al.^[178] presented a study of ligand-functionalized gold particles, which were arranged via DNA strands into “core-satellite” structures. Their systems consisted of a centered nanoparticle surrounded by smaller satellite nanoparticles. Linked by the DNA strands, the arrangement, stoichiometry, and shape of the colloids could be modified, thereby enabling precise modulation of ligand exposure and their interaction with cells.

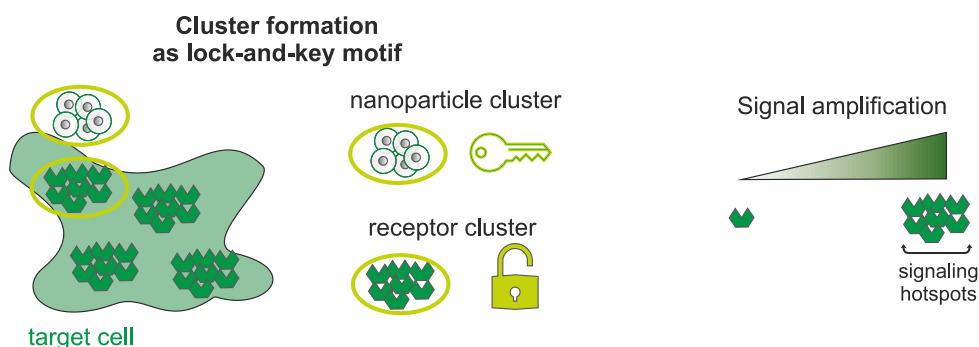


Figure 14 Schematic depiction of cluster formation as superordinate lock-and-key motifs.

When these results are incorporated in the evaluation of the lock-and-key model, cooperative effects between nanoparticles, respectively cross-dependencies between nanoparticle clusters and receptor clusters, should be included in the scope (Figure 14). Then, the “key” refers to the entire nanoparticle cluster or colloidal system, and the “lock” represents the reactively adaptive receptor cluster.

5. “The Door-Opener-Mechanism” or So-Called “Bystander Effect”

It was recently shown that the specific ligand-mediated interaction of nanoparticles with the cell surface has a “bystander effect” on nanoparticles, which themselves do not specifically interact with the cells and thus are not actively endocytosed.^[179,180] Figuratively speaking, the ligand-functionalized nanoparticles (FNP) work as “keys” in “opening the door”, respective cell, for the other, unfunctionalized, bystander nanoparticles (BNP) (Figure 15A).

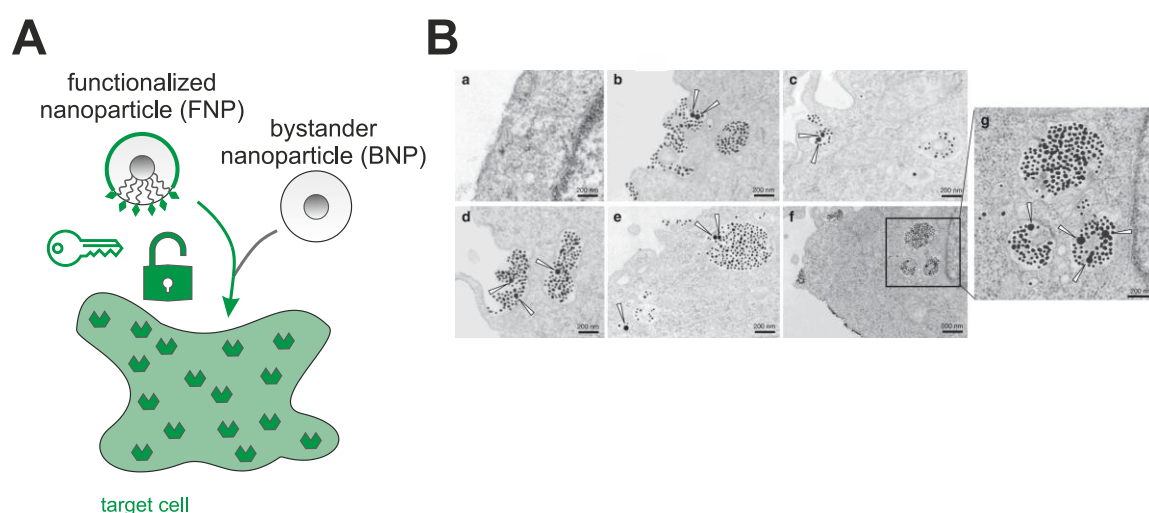


Figure 15 Uptake of bystander nanoparticles. (A) Schematic depiction of the bystander effect and the role of ligand-functionalized nanoparticles as “door openers” for the bystander nanoparticles. (B) Reprinted from Wei et al.^[179] Published Open Access, Nature Communication. The article is licensed under a Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>). Figure 3 was reprinted (<https://www.nature.com/articles/s41467-019-11631-w/figures/3>). TEM images of bystander gold nanoparticles (50-nm in diameter) alone (a) and in combination with ligand-functionalized gold nanoparticles (20-nm size) (b-g). (a) The bystander gold nanoparticles did not enter the cells in the absence of ligand-functionalized nanoparticles. (b-g) Endocytic structures of the bystander uptake.

The uptake of bystander cargo occurs naturally in cells as a non-specific and mostly receptor-independent process to absorb fluids and substances from the environment via macropinocytosis.^[181–183] Wei et al.^[179] employed TAT (transactivator of transcription) peptide modified nanoparticles to investigate whether these, following specific ligand-receptor-interaction with heparan sulfate proteoglycans (HSPGs) on the surface of target cell lines, influence the uptake of unfunctionalized BNPs. The BNPs themselves did not exhibit specific interaction with the cells and were therefore not endocytosed solely. The authors were able to demonstrate that the coadministration of TAT-functionalized nanoparticles stimulated the cellular uptake of the BNPs.

Control experiments were conducted, which confirmed that the bystander effect was not induced by a ligand transfer between the different nanoparticle types, and that the effect was mediated by specific ligand-receptor-interactions. TEM images of functionalized gold nanoparticles (20 nm in diameter), which were applied together with larger BNPs (50 nm in diameter), revealed that both nanoparticle types were internalized together in a macropinosome-like vacuole (Figure 15B). The authors concluded that the FNPs provided a “hitch ride” to the BNPs. The observed bystander effect was subsequently characterized using computer simulations to gain insights into the molecular mechanisms.^[180] The simulations revealed that the bystander effect is dependent on the interaction and affinity between both nanoparticle types and the cell surface, the properties and concentration of the FNPs, and the resulting membrane-curvature-mediated attraction between the FNPs and BNPs. The affinity of the BNPs to the membrane influences their diffusion on the cell surface and, consequently, their proximity to the FNPs. If their affinity is too low, the BNPs detach from the cell membrane or even do not bind to it. The simulations thus showed that the affinity of the BNPs must exceed a threshold value for the bystander effect to occur. The avidity of the FNPs for the target receptors influences the curvature of the membrane and is the driving force for the receptor-mediated endocytosis of the nanoparticles. It depends on the size and ligand valence of the FNPs. The correlation between increasing ligand valence of the FNPs and degree of internalization of the BNPs is highly non-linear: It initially increases with the degree of functionalization, then reaches a plateau, and subsequently decrease. Furthermore, the relative concentration between FNPs and BNPs also affects the cooperative interaction of both particle types with the membrane. Albeit, the described concentration effects are not associated with a cluster formation of the nanoparticles.

These results reveal that nanoparticles, which are incapable of engaging in ligand-mediated interactions with target cells themselves, can however benefit from the lock-and-key interactions of other nanoparticles.

6. Anti-Binding Effects and Regulated Multivalency

In a study on the murine herpesvirus 4, Delguste et al.^[33] demonstrated that the virus carries a glycoprotein (gp150) on its surface that negatively regulates viral binding to cells. Gp150 controls the number of interactions between the virus and the cell surface receptors, maintaining these interactions at a low level. It was proven experimentally that compared to gp150-deficient virions (gp150⁻), wild type virions exhibited reduced adhesion to target cells, as fewer and weaker interactions with the receptors were formed.^[33] Equipping a multivalent system with anti-binding moieties, which negatively regulate its multivalency, appears to be counterintuitive at first, as multivalency is frequently required to induce receptor clustering for subsequent endocytosis processes. However, despite the lower adhesion of the wild type virions to the host cells, their infectivity was higher. Other studies have shown that the diffusion of viruses and nanoparticles on the cell surface is associated with the bond valence and the targeted receptor type.^[184–186] Delguste et al.^[33] thus interpreted the binding-regulating effects in the context of an increased mobility of the herpes virions on the cell surface after initial adhesion. This would be coupled with a higher

probability of interacting with specific receptor patterns, thereby enhancing binding selectivity. Along this interpretation, the binding stoichiometry would be evolutionary optimized with respect to the diffusion rate of the virus on the cell surface. For functionalized lipid vesicles, Block et al.^[187] found their diffusion coefficient D across a binding moiety-positive bilayer to be proportional to $1/n$, with n indicating the number of bonds between the vesicle and the bilayer. This raises the question whether anti-binding effects, which negatively regulate the multivalency and thus likely increase the nanoparticles' mobility and binding selectivity, can be integrated into the design of ligand-functionalized nanoparticles.

Based on statistical mechanical modeling studies, Angioletti-Uberti^[47] proposed a concept to regulate the interaction between multivalent nanoparticles and cell receptors. The nanoparticles should carry targeting ligands and “protective receptors”, which can bind these ligands, on their surface. Figuratively speaking, the nanoparticles should be equipped not only with “keys” but also with the matching “locks” (Figure 16). The system exists in an equilibrium, controlled by the relative binding energies. An anti-binding effect against off-target receptors is created as these interactions are energetically less favorable compared to binding of the targeting ligands to “protective receptors”. The bonds between the ligands and the “protective receptors” dissociate in favor of binding events to target receptors, which have a higher binding energy. The author predicted that this form of interaction regulation between the receptors on the cell surface and ligands could enhance superselective behavior of nanoparticles.^[47]

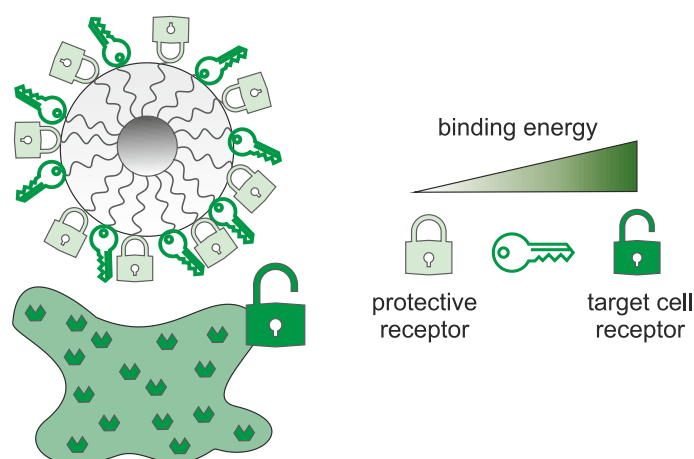


Figure 16 Regulated multivalence. The interaction between cells and nanoparticles, which carry not only ligands but also “protective receptors” on their surface.^[47]

Design strategies that induce switchable nanoparticle characteristics, such as sequential ligand presentation,^[41,134] ligand activation through enzymatic cleavage on the target cells^[188,189] or reversible regulation of bioactive ligands,^[190,191] also represent methods to regulate the multivalency of the interaction between nanoparticles and target cells. Albeit, they lack a genuine anti-binding element. Contrarily, the previously described coating with polymers, which possess antifouling

properties, confers anti-binding characteristics to the nanoparticles. However, these coatings do not regulate the nanoparticles' multivalency. An alternative approach to anti-binding effects is provided by the implementation of ligands into the nanoparticle design, which selectively inhibit their uptake into certain cells. In several studies, CD47, a transmembrane protein and "marker-of-self" in various species, or alternatively a structurally-related synthetic peptide have been coupled as ligands to the nanoparticle surface.^[192–194] CD47 impedes phagocytosis by its interaction with the signal regulator protein α , which is expressed by macrophages and dendritic cells.^[195] The phagocytosis of nanoparticles modified with CD47 is thus suppressed in these cells. For instance, the clearance of polymer nanoparticles targeted towards ICAM-1, a receptor overexpressed in many diseases, was significantly reduced by functionalizing them additionally with CD47.^[193] Their specific binding to the target receptor remained unaffected.

Aside from these few approaches, the topic appears to be largely untouched in research. However, it seems that not all options have yet been explored to implement anti-binding and multivalency-regulating elements in the design of ligand-functionalized nanoparticles. From the points outlined above, it becomes clear that the road to success does not lie in an uncompromising increase in nanoparticles' avidity. Extending the lock-and-key model by the concept of "anti"-keys therefore offers a promising approach and creates new perspectives for optimizing the selectivity of multivalent systems.

7. The "Key" and its Effect on the Host Cell: A View behind the "Lock"

Finally, a view behind the "lock" is warranted to explore the effects of the lock-and-key interactions on the target cells (Figure 17A). The interactions between ligands and cells trigger numerous intracellular signaling events, which significantly influence the functionality and state of the cell,^[196] e.g. adhesion, chemotaxis, inflammation, immune response, and secretion.^[197] The multivalent character of the binding between functionalized nanocarriers and cells can even amplify the effects of the ligand-receptor interaction.^[198] Agonistically functionalized nanoparticles elicit a cellular response in the cells to which they bind, whereas antagonistically functionalized nanoparticles exhibit partially inverse agonistic activity.^[199] Furthermore, the previously described receptor oligomerization, affected by the multivalent presentation of ligands, directly impacts cellular functions and signaling processes.^[177] It can contribute to an altered signal transduction and an increased sensitivity for ligands.^[15] Another crucial aspect, often overlooked, is the mechanical force that is exerted on the cell surface by the nanoparticle bonds. The enhanced binding strength and force of multivalent interactions have so far been primarily discussed in other contexts, such as for cell sorting^[177] or the intentional activation of mechanotransduction pathways.^[200] Mechanotransduction refers to the conversion of a physical force into a biochemical signal. For instance, ligand-functionalized magnetic nanoparticles were employed to specifically apply mechanical stimuli to cells and to initiate signaling processes.^[200] Mechanosensitive receptors enable cells to respond to environmental stimuli, which affect various cell functions, e.g. apoptosis, communication, contraction, cell differentiation, migration, proliferation. Mechanotransduction pathways play a crucial role in the pathogenesis of

various diseases and are thus of significant interest.^[201,202] In the context of biomedical nanoparticles, it is important to consider the risk of unintended mechanical effects that their binding could have on cells. Cells are furthermore interconnected through mechanical and biochemical linkages, which facilitates the transmission of signals between them. It becomes evident that the binding of functionalized nanoparticles to their host cells can significantly influence their functionality and, furthermore, impact the entire cell matrix through signal transmission between the cells. When the ligands on the nanoparticle surface are employed solely for active targeting, it is thus imperative to assess whether the pharmacological effects induced by the ligands align with the intended outcomes. In a recently published study by our group, we discussed ectoenzymes as alternative targeting structures.^[203] The PLGA/PLA-PEG nanoparticles functionalized with MLN-4760, a highly selective angiotensin-converting enzyme 2 inhibitor, showed a reliable target cell identification in coculture experiments. So far, target receptors are typically selected based on their expression levels on the host cells.^[60] The functionality and internalization dynamics of the receptors are thereby entirely disregarded.^[60] An alternative strategy would be to emphasize these aspects and to select receptors as targets by their cellular function and recycling rates. This could, on the one hand, enhance the uptake efficiency of nanoparticles. Receptors with a higher degree of internalization would therefore be particularly suitable as potential targets. Furthermore, many promising targets are located intracellularly. Consequently, the selection of appropriate receptors could positively influence the internalization, and subsequent intracellular distribution and targeting of the nanoparticles.^[204] On the other hand, the functionality of cells can be specifically modulated through their interaction with ligand-functionalized nanoparticles.^[196,205,206] In particular, Zhang et al.^[206] developed near-infrared-light-responsive nanomaterials that can be utilized as optogenetic tools, enabling the controlled activation and suppression of signaling cascades both *in vitro* and *in vivo*. Nanoparticles can thus serve not only as carriers for biomedical applications but also as a platform technology respective toolset for fundamental biological research. Their versatility enables their use in visualizing, quantifying, and even real-time control of cell signaling.^[205] Lee et al.^[205] and Hossen et al.^[207] have summarized the potential of this research field, which is still in its infancy, in insightful reviews.

Xie et al.^[75] presented an entirely different, innovative, and, in our opinion, highly intriguing perspective on cellular response processes to multivalent vectors. The authors discussed the mechanisms by which cells can alter their selectivity and sensitivity towards multivalent vectors, while adjusting their response threshold to maintain functionality. A sharp activation by stimuli is crucial for the survival of the organism. However, its effectiveness is limited if the activation threshold cannot be adjusted. The authors proposed the hypothesis that the up- and down-regulation of weak, non-specific interactions between receptors, that affect the inter-receptor attraction and thus their propensity to form clusters, might represent a cellular mechanism for modulating selectivity towards multivalent systems (Figure 17B). It is well established that receptor-receptor interactions can be modulated by processes such as receptor phosphorylation^[208] and receptor binding to adaptor proteins.^[209–211] The authors employed Monte Carlo Simulations to calculate the adsorption profiles of multivalent systems for varying receptor densities and different receptor-receptor interactions. They demonstrated that a variation in the inter-receptor attractiveness, and consequently in the

cluster formation probability, influences both the binding threshold for multivalent systems and the binding selectivity. It can thus contribute to multivalent superselectivity. They explained this by the fact that the binding of multivalent systems becomes a cooperative process on the cellular site by receptor-receptor interactions.

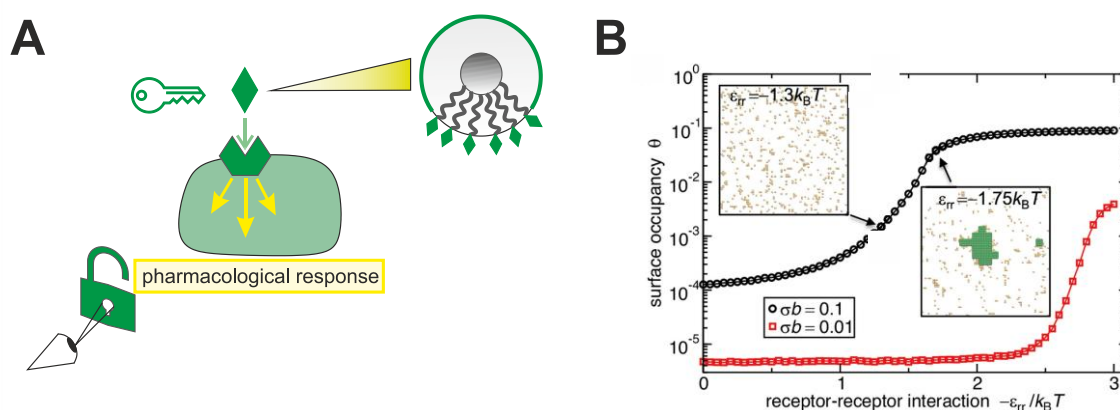


Figure 17 Effects of multivalent binding to target cells. (A) Schematic illustration of pharmacological response after ligand binding. (B) Reprinted adapted from Xie et al.^[75] Published Open Access. Proceedings of the National Academy of Sciences of the United States of America. The article is licensed under a Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>). Figure 3 is reprinted in part (<https://www.pnas.org/doi/10.1073/pnas.2417159122>). Correlation of surface occupancy (θ) (= surface fraction of the adsorbed particles) and receptor-receptor interaction ($-\epsilon_{rr}/k_B T$) with the receptors being indicated as orange spheres and the nanoparticles as green spheres at two receptor densities (σb).

Based on the above, it becomes evident that the challenge and opportunity for the design of multivalent nanocarriers lies in the fact that receptors are not merely counterparts in a lock-and-key interaction but furthermore possess intrinsic functionalities. Gaining broader understanding on cellular processes and emphasizing the functionalities of the cell receptors could therefore represent the next major advancement in the development of successful multivalent systems.

Conclusion

In our view, applying the metaphor of the lock-and-key principle, as introduced for the interaction between a single ligand and its receptor, to ligand-functionalized nanoparticles represents an overly simplistic understanding of their multivalent interaction with targeting structures. Our detailed analysis reveals that the model needs to be expanded to better represent the complexity of these interactions. Due to their high surface energy, nanoparticles exhibit significant interaction potential even without ligand-functionalization. Moreover, the assumed ligand valence does not necessarily correspond to the actual number of available ligands on the nanoparticle surface. Most important, a single ligand cannot be considered to represent the binding motif. Instead, the concept of binding “keys” must be expanded to encompass (geometric) binding patterns that are (adaptively) reflected on the cell surface by receptor patterns. Nanoparticles can also exhibit cooperative behavior in their binding to cell surfaces and subsequent internalization. This further broadens the understanding of binding motifs and raises questions regarding the fundamental concentration dependency of interaction and uptake processes between nanoparticles and cells. – A topic that has received little attention in the literature thus far but is gaining relevance in this context. Furthermore, observations from nature demonstrate that multivalent binding patterns derive advantages in their selectivity and specificity through the influence of anti-binding elements. When applied to nanoparticles, this prompts the question of how anti-binding and multivalency-regulating elements can be integrated into their design. On the cellular side, the understanding of “lock”-motifs must be broadened to encompass receptor clusters. These may already exist on the cell surface or emerge through interaction with multivalently presented ligands. Moreover, the receptor clusters could serve as cellular control modules for response regulation. In our opinion, it is therefore outdated and insufficient to select targeting structures for nanoparticles solely based on their expression levels in the target tissue. Instead, greater emphasis should be placed on cellular processes and on the mechanisms of nanoparticle-cell interactions.

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Chapter 2

Goals of the Thesis

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Nanoparticles have been investigated for decades as a promising platform technology for application as drug delivery systems.^[1,2] Their significant potential is recognized in the ability to protect their cargo from external influences and to facilitate targeted transport to the site of action.^[3,4] The latter is attempted to be achieved by engineering the nanoparticles' surface with ligands, which bind specifically to receptors on the target cells.^[5,6] This strategy is known as active targeting.^[7] By leveraging the differences in the receptor expression patterns between target and off-target cells and establishing tailored targeting concepts, targeting selectivity is aimed to be attained.^[8] Viruses, which are evolutionary optimized towards their targets, frequently serve as a role model for the approached targeting strategies.^[9–11] However, while the functionalization of nanoparticles with ligands demonstrates outstanding success in terms of their targeting efficiency *in vitro*, they still fail to meet expectations *in vivo*.^[12] This can partially be attributed to the fact that the complexity of the binding of functionalized nanoparticles towards their target cells is not yet fully captured (**Chapter 1**). Consequently, the active targeting concepts could not be perfectly adapted. However, an equally critical factor is that nanoparticles are quickly recognized as foreign material and sequestered by immune cells after administration *in vivo*.^[13–15] Their clearance from the blood circulation typically occurs within minutes.^[16] This significantly shifts their distribution towards off-target tissues, e.g. the liver. While *in vitro*, their targeting efficiency primarily depends on the clear differentiation between target and off-target cells based on receptor expression patterns, *in vivo* it is predominantly influenced by their recognition through the immune system. This contributes to the fact that, despite sophisticated ligand-based targeting concepts, nanoparticles currently achieve only minimal targeting efficiency and are almost exclusively applied systemically.

The goal of the present work was to address this challenge from two perspectives. Firstly, the specific and non-specific interactions between ligand-functionalized model nanoparticles and their target cells were characterized (**Chapter 3 and Chapter 4**). This was aimed at contributing to a more profound understanding of the binding mechanisms, thereby laying the foundation for rational design strategies for nanoparticles. Secondly, a complementary approach was devised to protect the nanoparticles from detection by the immune system and thus to modulate their target/off-target distribution. The coadministration regime developed for this purpose was specifically optimized to enable the subcutaneous administration of the nanoparticles (**Chapter 6**). A procedure was established to label the nanoparticles radioactively with technetium-99m (**Chapter 5**), to allow for the examination of their biodistribution and target efficiency *in vivo*.

Polymeric core-shell nanoparticles, which are composed of a poly(D,L-lactide-co-glycolide) PLGA core with attached poly(ethylene glycol)-b-poly(D,L-lactide) PLA-PEG block copolymer, were employed for the investigations. The hydrophilic PEG chains extend from the hydrophobic PLGA/PLA core forming the nanoparticle shell. From the core-shell structure, a high degree of modularity in terms of the nanoparticles' surface modification emerges.^[10] This allows the realization of complex targeting concepts,^[11,17,18] which makes the nanoparticles a promising platform and an interesting model system. To examine the binding mechanisms between these model nanoparticles and cells, the nanoparticles' surface was functionalized with losartan carboxylic acid (EXP3174), an antagonist

at the angiotensin-II type 1 receptor (AT1R).^[19] It has been demonstrated in previous studies that a sufficient surface functionalization with the ligand enables the effective anchoring of the nanoparticles to the surface of receptor-positive cells.^[11] The antagonistic effect of EXP3174 at the receptor furthermore promotes an endocytosis inhibition of the nanoparticles.^[19] This facilitates the selective detection of nanoparticle binding to the cell surface, clearly separated from their cellular uptake. The interaction of EXP3174-functionalized nanoparticles with receptor-positive rat mesangial cells^[17] was characterized at the level of ligand-receptor binding and non-specific adhesion.

A comparison with the spike density of viruses reveals that engineered nanoparticles are usually equipped with significantly more binding moieties on their surface.^[20] This could be a key factor contributing to the considerably lower binding selectivity of nanoparticles. To quantitatively characterize the specific ligand-mediated interactions of nanoparticles with their target cells, an experimental approach was established to determine the fraction of binding ligands on the nanoparticles' surface (**Chapter 3**). Therefore, the number of binding ligands was defined as a function of the number of target receptors on the cell membrane relative to the number of nanoparticles attached to the cell. This correlation can be considered valid assuming certain conditions are met. All variables of the formulated equation were progressively determined through experiments. The calculated average number of binding ligands per nanoparticle was subsequently interpreted within the biological context of the target cell line.

Additionally, the model nanoparticles were assessed for their potential to engage in non-specific interactions with the target cells (**Chapter 4**). The adhesion of the nanoparticles towards cell membranes was analyzed as a function of their surface charge. Therefore, binding studies of functionalized nanoparticles, which carried differently charged PEG chains in their shell, were conducted on rat mesangial cells. To examine the potential of the hydrophobic ligand to intercalate (non-specifically) into the cell membrane, the adhesion of EXP3174-functionalized and unfunctionalized nanoparticles towards supported lipid bilayers was compared. Supported lipid bilayers were chosen as a well-controlled model system.^[21,22] It facilitates the investigation of the interaction between nanoparticles and biomembranes, while eliminating effects mediated by other cell membrane components (e.g. glycoproteins and carbohydrates).

To make the nanoparticles accessible for real-time tracking in vivo, they were labeled radioactively. Therefore, pertechnetate was reduced using appropriate reducing agents and embedded as technetium-99m into the core-shell structure of the nanoparticles. The success of the radiolabeling process and the presence of radiochemical contaminants were examined. Radio-thin layer chromatography is a commonly employed method for assessing the radiochemical purity of radiopharmaceuticals.^[23] Its application to radiolabeled nanoparticles is widely accepted.^[24–27] In **Chapter 5**, the general transferability of thin-layer chromatography as a quality control tool between different settings and nanoparticle types was evaluated.

Following their administration in vivo, the bioavailability and targeting efficiency of nanoparticles is dramatically affected by their sequestration by immune cells.^[13,14] Consequently, their applicability is mostly restricted to systemic administration as the route of application. In **Chapter 6**, a

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complementary approach to nanoparticle engineering was explored by developing a coadministration regime with decoy particles. The aim was to saturate the uptake capacity of immune cells with decoy particles, thereby protecting the nanoparticles from endocytosis and modulating their target/off-target distribution. Several coadministration regimes with both lipid and polymer decoy particles and chloroquine, an endocytosis inhibitor,^[28,29] were investigated for their effect on uptake inhibition of nanoparticles into macrophages. The application regimes were furthermore designed to enable subcutaneous administration as a novel application route for nanoparticles.

In summary, the fundamental goal of the present work was to foster the understanding of nanoparticle-cell interactions and develop strategies aimed at enhancing their targeting efficiency and modulating their distribution.

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Chapter 3

The Quantity of Ligand-Receptor Interactions between Nanoparticles and Target Cells

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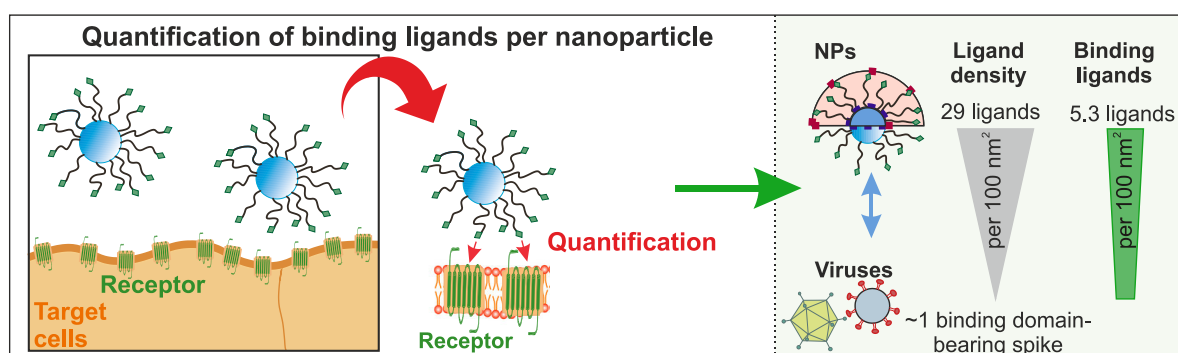
K. S. (first author) designed and performed the research, analyzed the data und wrote the manuscript. All experiments and data that are not explicitly assigned to the contribution of other authors in the following are to be attributed to K.S.. S. B. provided scientific input to the project. O. Z. and F. J. B. contributed to the estimation of the receptor density in clathrin coated pits and generated Figure 8C. M. K. synthesized the radioligand, generated Figure S10A, wrote the Methods section describing the radioligand synthesis and characterization and provided scientific input to the quantification of receptor numbers on the target cell line. R. W. contributed to the acquisition of the TEM images and reviewed the final manuscript. A. G. supervised the project, provided scientific input during the complete process, supported the manuscript preparation and reviewed the final manuscript. The ICP-MS measurements were conducted by the INDIKATOR GmbH (Wilhelm-Muthmann-Str. 15, 42329 Wuppertal) on a contractual basis.

New Concept Statement

In our work, we discuss the number of specific ligand-receptor interactions between engineered nanoparticles and their target cells as a decisive factor for cell specificity and selectivity in comparison to the spike density of viruses. Interactions of nanoparticles carrying an antagonist to the angiotensin II receptor type 1 with receptor positive cells showed that only a minority of the ligands in the densely functionalized nanoparticle corona were involved in specific interactions with the target cells. We were able to reveal for the first time that the number of specifically binding ligands on the surface of nanoparticles is of the same order of magnitude as the spike density of viruses that are evolutionary optimized. By considering the receptor structure and surface morphology of the target cell line, we were able to plausibly explain the result in the specific biological context. This reveals further optimization potential for engineered nanoparticles in terms of ligand density and structuring. By presenting an experimental approach to quantify the number of binding ligands, we lay the foundation for design strategies that aim at optimizing the number of ligands, which could be the next step in the development of active targeting concepts for biomedical nanoparticles.

Abstract

Achieving high target cell avidity in combination with cell selectivity are fundamental, but largely unachieved goals in the development of biomedical nanoparticle systems, which are intricately linked to the quantity of targeting functionalities on their surface. Viruses, regarded as almost ideal role model for nanoparticle design, are evolutionary optimized. They cope with this challenge bearing an extremely low number of spikes, and thus binding domains, on their surface. In comparison, nanoparticles are usually equipped with more than an order of magnitude more ligands. It is therefore obvious that one key to increasing the nanoparticles' efficiency in terms of avidity and selectivity lies in optimizing their ligand number. A first step along this way is to know how many ligands per nanoparticle are involved in specific binding with target cell receptors. This question is addressed experimentally for a block copolymer nanoparticle model system. The data confirms that only a fraction of the nanoparticles' ligands is involved in the binding processes: With a total ligand valence of 29 ligands/100 nm² surface area a maximum of 5.3 ligands/100 nm² are involved in specific receptor binding. This corresponds to an average number of 251 binding ligands per nanoparticle, a number that can be rationalized within the biological context of the model system.



Graphical Abstract

Introduction

Polymer nanoparticles, which are investigated as innovative platforms for drug delivery and diagnostics, are often equipped with small molecules or peptides as ligands on their surface to achieve high specificity and selectivity for their target sites.^[1] To further enhance their efficiency, nature is frequently taken as an example. In particular, viruses, adorned with receptor binding domains on their spikes as evolutionarily optimized recognition structures, serve as excellent role models.^[1,2] There are numerous research strategies that aim to mimic the recognition mechanisms of viruses on cell surfaces.^[3] However, while the spike density is strongly regulated in viruses, engineered nanoparticles are usually equipped with a significantly higher number of ligands.^[4] Evidently, the initial contact between the engineered nanoparticles and cells is decisive for recognition and attachment and therefore determines their success or failure.^[5] Thus, optimizing the number of ligands on their surface is of utmost importance to balance target cell avidity and selectivity.^[6] Still, a close look at the literature reveals that there is a knowledge gap about how many ligands per nanoparticle are involved in the anchoring process and specifically bind to receptors.

Attempts have been made to characterize the mechanisms underlying particle association at the cell surface and consequently affecting the number of binding ligands.^[5,7–9] It is known from thermodynamic considerations that the avidity of the particles is increased by processes and factors that increase the enthalpy gain in the system, e.g. the specific binding of a ligand to its receptor.^[10,11] Conversely, the loss of entropy that occurs in multivalent bonds due to the reduction of degrees of freedom has a negative regulating effect.^[12] Another important parameter is the geometric congruence of the two counterparts. We have recently examined the role of the geometry and curvature of clathrin-coated pits on the avidity of nanoparticles by increasing the number of potential ligand-receptor contacts.^[13] Mathematical and computational models provide further insights into the binding processes even at the level of individual receptors and deliver preliminary information regarding the stoichiometry of binding.^[8,12,14] Farokhirad et al.^[12] presented a computational approach to predict the number of binding ligands per nanoparticle for a multivalent system depending on particle rigidity and membrane excess area. In their study on the biomimetic design of nanoparticles, Yuan et al.^[15] found that the sweet spot of the ligand number for optimal uptake conditions of virus particles is in the range of 10 to 100 ligands per virus particle, regardless of particle size. Loading the particles with ligands beyond the optimum leads to intensified competition for receptors, which are a limited source on the cell surface. A prolonged endocytic time and lower cellular uptake of the particles is the consequence. Oppositely, the concept of multivalence fails when the number of ligands drops below a system-specific threshold,^[16] as the entropy loss predominates.^[14,15] This underlines that adjusting the number of ligands to the number actually required for the anchoring process could significantly increase the efficiency of the engineered nanoparticles, also in terms of better uptake rates and optimized uptake kinetics.^[15–18] Computational studies have the distinct advantage of well-defined and controlled experimental conditions,^[8] because they are based on a limited number of assumptions. The resulting mathematical and thermodynamic models are

consequently associated with simplifications that cannot fully capture the complexity of real conditions.^[8,19] Quantifying the number of binding ligands per nanoparticle in further experimental studies, would therefore be of paramount relevance for the development of competitive multivalent nanoparticle designs.^[19]

The intention of our study was to provide an experimental approach for the quantification of ligand-receptor interactions between functionalized nanoparticles and target cells, which copes with the complexity of the system. Core-shell nanoparticles composed of poly(D,L-lactide-co-glycolide) (PLGA) and a poly(ethylene glycol)-b-poly(D,L-lactide) (PLA-PEG) block copolymer were selected as well-established model.^[1,2] The nanoparticles were functionalized with losartan carboxylic acid (EXP3174), a high-affinity antagonist at the angiotensin-II type 1 receptor (AT1R). The ligand leads to an endocytosis inhibition of the nanoparticles and therefore enables the detection of surface-related effects^[20] on AT1R expressing rat mesangial cells (rMCs).^[1] To make the polymer nanoparticles accessible for quantitative detection on the cellular matrix, we implemented selenium^[21] into the nanoparticles as a tag for inductively coupled plasma mass spectrometry (ICP-MS).^[22,23] By defining the average number of binding ligands per nanoparticle as a function of half of the number of target receptors on the cell surface divided by the number of nanoparticles attached to the cell membrane, we were able to formulate an experimentally solvable equation. The equation requires the assumption that exactly half of all receptors on the cell surface are occupied by ligands. This can be achieved experimentally if the applied nanoparticle concentration is equal to their equilibrium inhibition constant, K_i .^[24] Through successive experiments we were able to calculate the average number of binding ligands and furthermore to draw a comparison with viral systems. With our study, we aimed to contribute to a systematic understanding of the interactions between nanoparticles and cells at the level of individual receptors and to a rational design of target cell selective nanoparticles.

Results

Depiction of the Underlying Model

The engineered nanoparticles chosen as a model for this study were designed as PLGA/PLA-PEG core-shell nanoparticles with a rigid, tethered and spherically enclosed morphology and functionalized with EXP3174 as ligand (Figure 1, Figure S1, Figure S2, Figure S3).^[12,25] EXP3174 was synthesized with a purity of > 95 % and coupled to the PLA_{10K}-PEG_{5K} block copolymer with a coupling efficiency of at least 98 % (Figure S4, Figure S5). In a previous study, we have evidenced a 25 % surface functionalization with EXP3174 as the optimal ligand density to ensure sufficient AT1R binding and anchoring of the nanoparticles to the cell surface^[2] and the degree of functionalization was adopted in the present study (Figure 1, Figure S6, Figure S7).

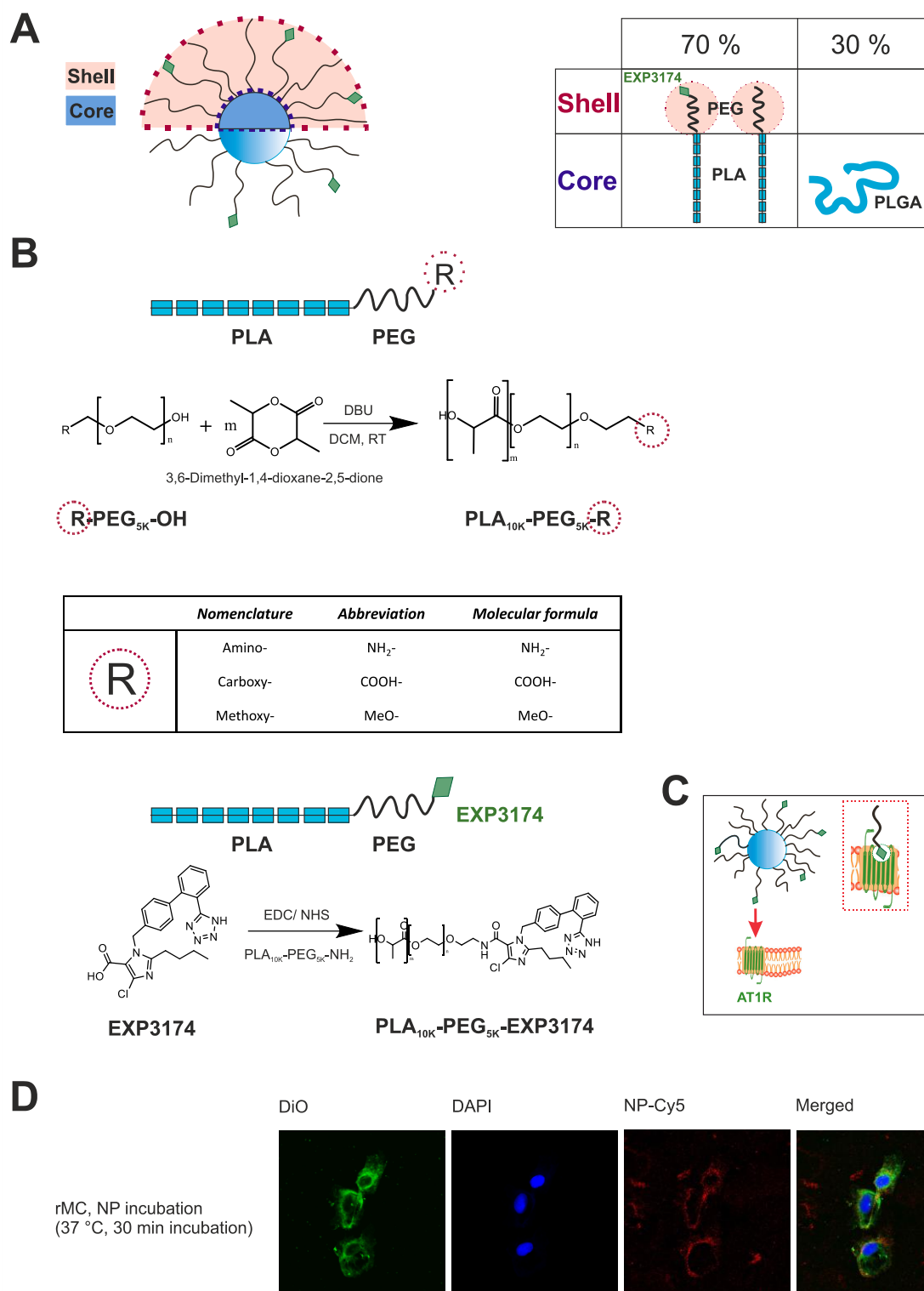


Figure 1 Illustration of the design and targeting concept of the polymer core-shell nanoparticles and their cellular localization. (A) Nanoparticles were obtained by nanoprecipitation of hydrophobic PLGA blends with amphiphilic PLA-PEG block copolymer. By removal of the organic solvent, the water-insoluble polymers self-assemble into core-shell structured nanoparticles. PLGA and PLA are miscible and aggregate in the nanoparticle core due to their higher hydrophobicity,^[28,29] with the hydrophilic PEG chains extending in a brush-like corona from the core. PLGA and PLA-PEG were used in the ratio 30:70 (m/m). The use of PLGA as core material enables the incorporation of fluorescent dyes, e.g. CY-5, into the core of the nanoparticles for flow cytometry and microscopic analysis. To create a rigid, tethered and spherically enclosed morphology, both, the ligand-bearing, and the carboxy-terminated spacer chains, which are intended to prevent steric hinderance

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between the ligand molecules on the nanoparticle surface,^[30,31] were synthesized as block copolymer chains of the same molecular weight (PEG_{5K}-PLA_{10K}). (B) Schematic reaction equation of ring-opening polymerization, where R indicates the different terminal charges of the PLA-PEG chains, and reaction equation of PLA-PEG chain functionalization with the ligand EXP3174. (C) Illustration of the active targeting concept of the EXP3174-functionalized nanoparticles at the AT1R on the surface of rMCs. EXP3174 acts as a high-affinity antagonist at the AT1R. (D) Confocal laser scanning microscopy images of rMCs incubated with CY-5-labeled 25 %-EXP-NPs (red) for 30 min at 37 °C (5 % CO₂). Cell nuclei were stained with DAPI (blue) and cell membranes were stained with DiO (green). The binding experiment showed that the nanoparticles bound to the cell surface of the rMCs but were not internalized.

Elemental selenium^[21] was embedded into the nanoparticle core to serve as an ICP-OES and ICP-MS tag for subsequent nanoparticle quantification in the cellular matrix over a wide concentration range (Figure 2A, Figure S8).^[22,23] Elemental selenium is unstable in aqueous solution;^[21] however, it can be stabilized as an amorphous dispersion through the addition of polymers when produced via in situ reduction of water-soluble sodium selenite.^[21,26,27] Both nanoparticle types, selenium-tagged nanoparticles (25 %-EXP-NPs_selenium-containing) and unlabeled polymer nanoparticles (25 %-EXP-NPs), were compared in terms of their morphology (Figure 2B), surface charge and size distribution (Figure 3, Figure S8) to characterize the incorporation of the selenium tag for its effects.

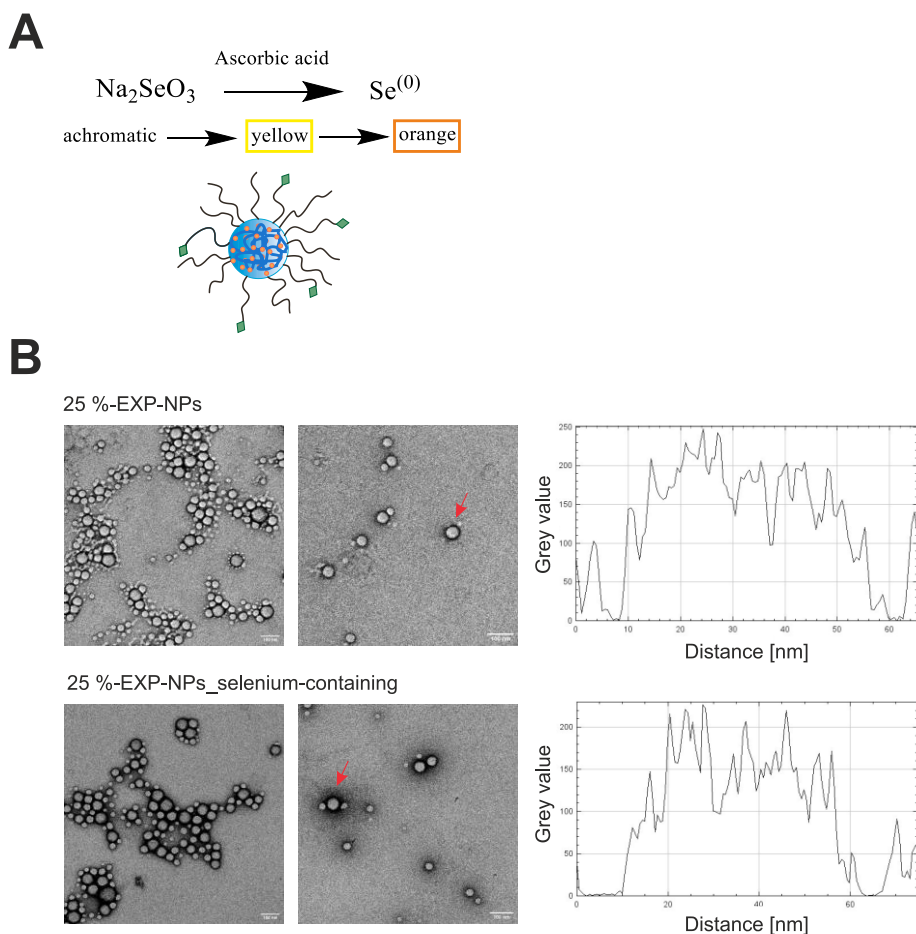


Figure 2 Implementation of selenium as ICP-OES and ICP-MS tag in the polymer matrix and characterization of the morphology of selenium-containing and selenium-free nanoparticles. (A) Schematic depiction of the redox reaction of sodium selenite with ascorbic acid to elemental selenium and the stabilization of the elemental selenium in the polymeric matrix. The progress of the reaction can be

recognized by a color change from achromatic to orange.^[21] (B) Representative TEM images of selenium-free (25 %-EXP-NPs) and selenium-containing nanoparticles (25 %-EXP-NPs_selenium-containing) after negative staining with uranyl acetate (scale bar: 100 nm). Shape and integrity of the nanoparticles were confirmed. The grey value of two exemplary nanoparticles, which are comparable in size and marked with red arrows, is plotted. The selenium-containing nanoparticles showed a darker frame, compared to the unlabeled polymeric nanoparticles, indicating an increased atomic mass associated with the incorporated selenium, as more electron beam is absorbed in these areas.

Size distribution data were collected using three analytical methods based on different measurement principles (Table S1). DLS and NTA measurements provide hydrodynamic diameters, while TEM-based analyses measure the nanoparticles without their hydration layer.^[32,33] The obtained size distribution curves were evaluated with regard to the expected total number of ligands on the nanoparticle surface, hereafter referred to as mean ligand valence (Figure 3, Table S1). To form a mathematical relationship between the measured particle size and the mean ligand valence, the nanoparticles shape was assumed to be a perfect sphere. Their surface area (O_{NP}) was subsequently calculated from the (hydrodynamic (dh)) diameter (d), as shown in equation 1.

$$O_{NP} = \pi \cdot (dh)^2$$

eq. 1

The mean ligand valence per nanoparticle was assessed by dividing O_{NP} by the surface area occupied by each PEG chain (S) in the nanoparticle shell.^[34,35] We considered this justified under the assumption that S is a circular area, and that the polymer distribution is homogeneous.

$$S = \frac{6 \cdot M_{PEG}}{dh \cdot N_A \cdot f \cdot \rho_{NP}}$$

eq. 2

According to equation 2, S is defined by the molecular weight of a PEG chain (M_{PEG}), which is related to the (hydrodynamic) diameter of the nanoparticles (dh), the Avogadro constant (N_A), the mass fraction of PEG in the blends of the PLA_{10K}-PEG_{5K} block copolymer (f), and the density of the nanoparticles (ρ_{NP}).^[35] A density of 1.25 g cm⁻³ was assumed as a rough estimate for the density of the nanoparticles. The value corresponds to the density of nanoparticles composed of PLA-g-PEG blends.^[36]

A coefficient was introduced to account for the fact that only 25 % of the PEG chains were functionalized with ligands. The mean ligand valence (N_L) was therefore described as a function of the nanoparticles size as follows (equation 3):

$$N_L = 0.25 \cdot \frac{O_{NP}}{S}$$

eq. 3

The analysis of the collected data revealed a significantly differing mean ligand valence, shown as mean $\pm$ SD and mode (Table S1), depending on the underlying measurement method. The ligand

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density on the nanoparticle surface could be calculated from this in an order of magnitude from 10 to 29 ligands per 100 nm² surface area.

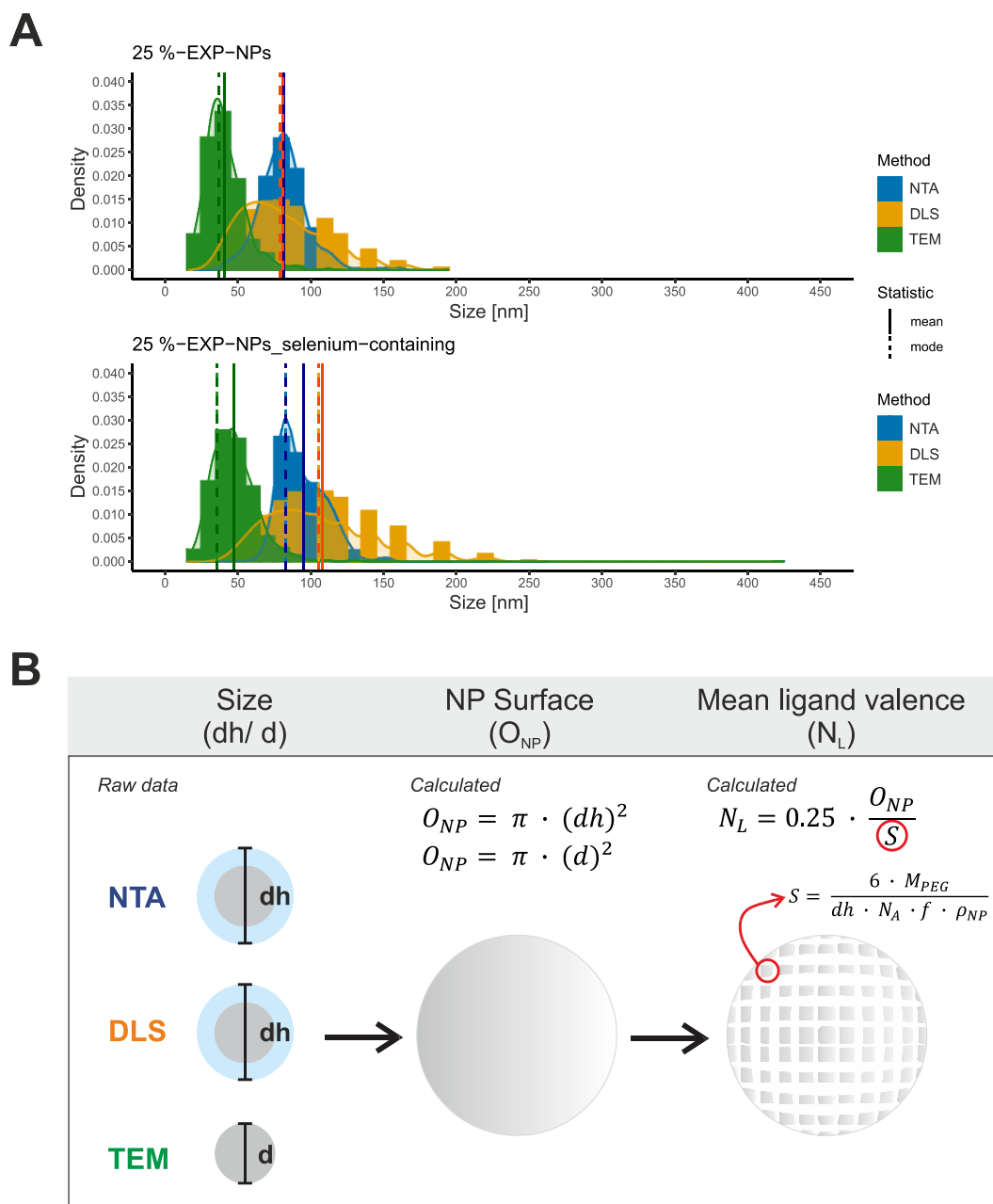


Figure 3 Characterization of size and ligand distribution of the model nanoparticles. (A) Size distributions of 25 %-EXP-NPs and 25 %-EXP-NPs_selenium-containing plotted as a function of the measurement method. Size values obtained by NTA, DLS, and TEM measurements were compared. NTA and DLS raw data (DLS data as intensity percent) were obtained in a triplicate measurement ($n = 3$). The DLS data was furthermore analyzed as volume percent (Figure S9). The size of 500 nanoparticles was measured on six independent sections of each TEM grid. The mean and modal values of the distribution curves were highlighted in color of the curves as solid and dashed lines in the figure. (B) Illustration of the assumed mathematical relationship between the measured nanoparticle (hydrodynamic) diameter and the calculated nanoparticle surface area and mean ligand valence. The parameters considered were the surface that each PEG chain occupies on the nanoparticle surface (S), the molecular weight of the PEG chain (M_{PEG}), the mass fraction of PEG in the PLA-PEG blends (f) and the assumed density of the nanoparticles (ρ_{NP}).

To proceed with determining the average number of binding ligands of the described model nanoparticles, the mean value of specifically binding ligands (N_{bound}) was defined as a function of the occupied receptors (N_{oc}) on the target cell surface relative to the number of nanoparticles (N_{NP}) attached to the cell membrane as shown in equation 4 (Figure 4).

$$N_{bound} = \frac{N_{oc}}{N_{NP}}$$

eq. 4

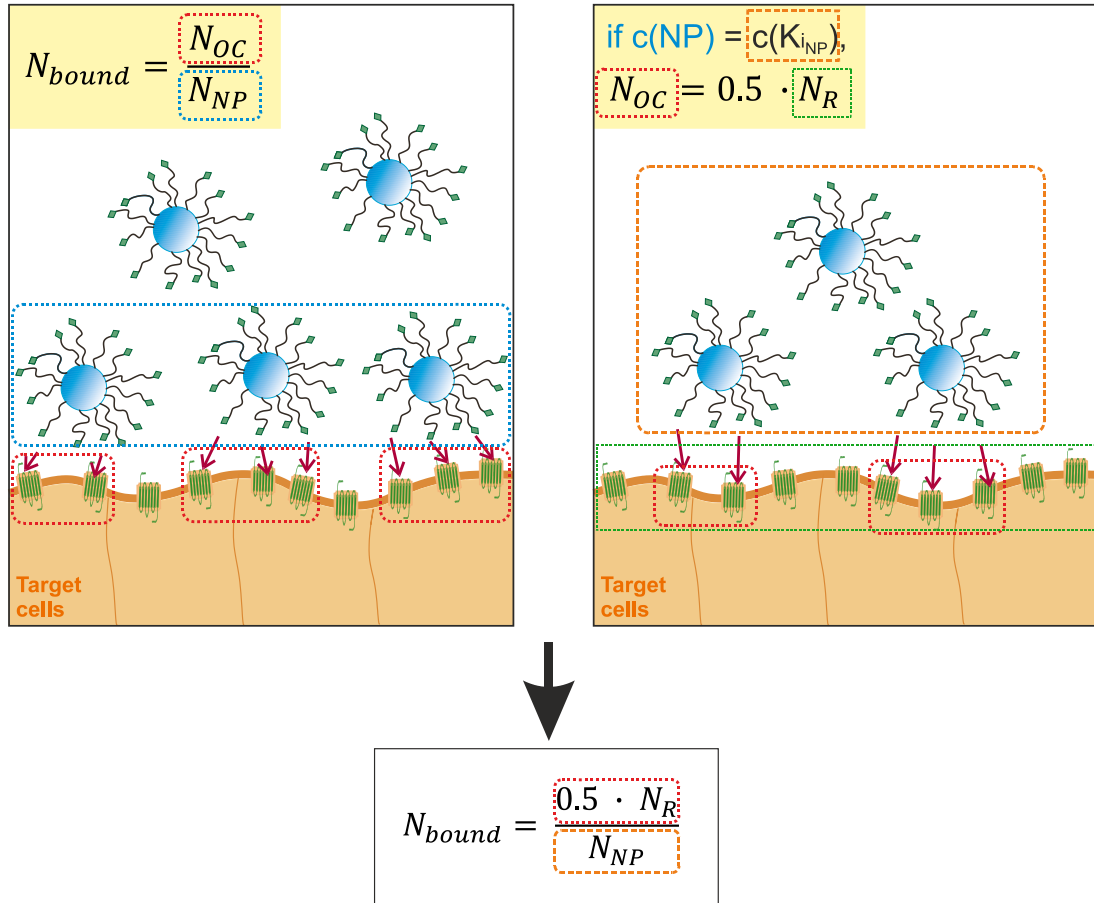


Figure 4 Illustration of the assumptions underlying the formula for calculating the average number of binding ligands per nanoparticle.

At the concentration of the K_D value, half of the maximum specific binding sites (N_R) are occupied by ligands.^[37,38] This correlation also applies, if the number of nanoparticles added to the system corresponds to the concentration of their K_D value. This allows us to define N_{oc} as a function of the total number of available receptors (N_R) on the cell membrane (equation 5).

$$N_{oc} = 0.5 \cdot N_R$$

eq. 5

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Likewise, N_{oc} can be defined as a function of N_R in the region of receptor saturation, when all available receptors on the cell membrane are occupied.^[39] Then the relationship $N_{oc} = N_R$ holds. However, we assumed that this would have experimental disadvantages, as saturation requires the addition of a unphysiological excess of nanoparticles.

The previously defined expression for N_{oc} as a function of half of N_R can be inserted into equation 4 as shown in equation 6.

$$N_{bound} = \frac{0.5 \cdot N_R}{N_{NP}}$$

eq. 6

The number of binding ligands is then defined as a function of half of the number of target receptors on the cell surface allocated to the number of nanoparticles attached to the cell membrane. We considered this justified if the applied nanoparticle concentration is equal to their K_D value or, in the case of the antagonistic functionalized nanoparticle, to their equilibrium inhibition constant, K_i .

Equation 6 is based on variables that can be obtained through successive experiments as described in the following.

Determination of the Average Number of Binding Ligands per Nanoparticle

First, the average number of AT1R expressed on the surface of the target cell line (N_R) was determined by saturation binding studies with the tritium-labeled Ang-II derivative [³H]UR-MK292. The maximal number of binding sites (B_{max}) was extracted from the fitting parameters of specific binding and converted to the receptor number related to the total number of rMCs used in the experiment (Figure 5, Figure S10). We found N_R to be $22,000 \pm 1500$ AT1R per cell (mean $\pm$ SD from four independent experiments).

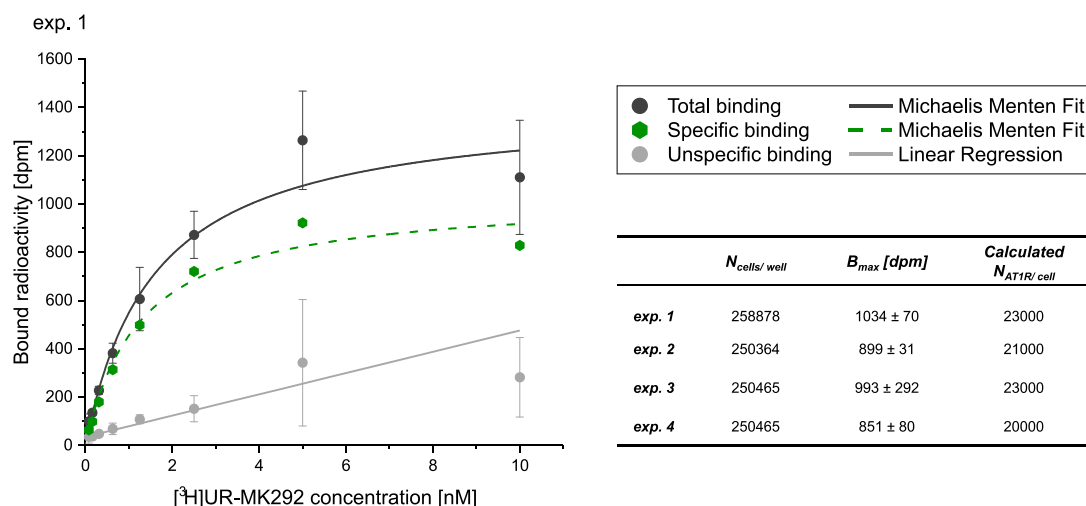


Figure 5 Radiochemical saturation binding studies with the tritium labeled Ang-II derivative $[^3\text{H}]\text{UR-MK292}$ to determine the average number of AT1R per cell on rMCs. Data were obtained from four independent experiments (exp. 1 – 4), with data from experiment 1 shown as a representative example (mean $\pm$ SD; $n = 3$). The obtained results are presented in the table with $N_{\text{cells/well}}$ indicating the counted number of cells per well in the experiment, B_{max} [dpm] indicating the maximum recorded radioactivity in the total binding experiments and calculated $N_{\text{AT1R/cell}}$ indicating the calculated average number of AT1R per cell. Please refer to Figure S10 for experiments 2 – 4.

To determine the K_i value of the EXP3174-functionalized nanoparticles, the fura-2 AM based Ca^{2+} mobilization assay established by Grynkiewicz et al. was utilized.^[40,41] The assay was selected as its results are unaffected by non-specific binding to the cell surface or receptors. This has been previously demonstrated by our group by proving that both, non-functionalized nanoparticles on AT1R expressing cells and EXP3174-functionalized nanoparticles on off-target cells, showed no activity in the assay.^[2] The K_i value was derived from the avidity of the particles, which was measured by inhibiting the AT1R under competition with the agonist Lys-Ang-II. The obtained data sets were plotted as binding curves normalized to the highest value in each dataset. The extracted IC_{50} values were averaged for each nanoparticle type (25 %-EXP-NPs: 0.50 ± 0.09 pM; 25 %-EXP-NPS_selenium-containing: 0.70 ± 0.07 pM; mean $\pm$ SEM from five independent experiments) (Figure 6A and B, Figure S11, Figure S12). The derived IC_{50} values were found to be statistically equal (Welch's two-sample t-test: $\text{Pr}(> t) = 0.1039$). This underscores that the selenium tag had no influence on the activity of the nanoparticles on the target site. The IC_{50} values were converted to the corresponding equilibrium inhibition constant, K_i , using the Cheng-Prusoff transformation,^[42] where $[L]$ is the competing agonist concentration and K_D is its equilibrium dissociation constant (equation 7):

$$K_i = \frac{IC_{50}}{1 + \frac{[L]}{K_D}}$$

eq. 7

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The K_D value of Ang-II for the AT1R is published to be in the range of 1 nM.^[43] However, since the assay used to determine the IC_{50} value of the EXP3174-functionalized nanoparticles is coupled to the Ca^{2+} signal at the receptor, we decided to record the K_D value of the competitive agonist under the same conditions. The effect (E) of the Lys-Ang-II on the receptor was fitted as a function of ligand concentration ($[X]$) (let X be Lys-Ang-II) in a classical saturation binding model^[13,44] as shown in equation 8.

$$\frac{E}{E_{max}} = \frac{[X]}{[X] + K_D^x}$$

eq. 8

The obtained data were averaged to a mean K_D value for Lys-Ang-II of 51.9 ± 20.4 nM (mean $\pm$ SEM). Subsequently, the averaged K_i value of EXP3174-functionalized nanoparticles was calculated to be 0.14 pM for the unlabeled polymer nanoparticles and to be 0.19 pM for the selenium-tagged nanoparticles according to equation 7.

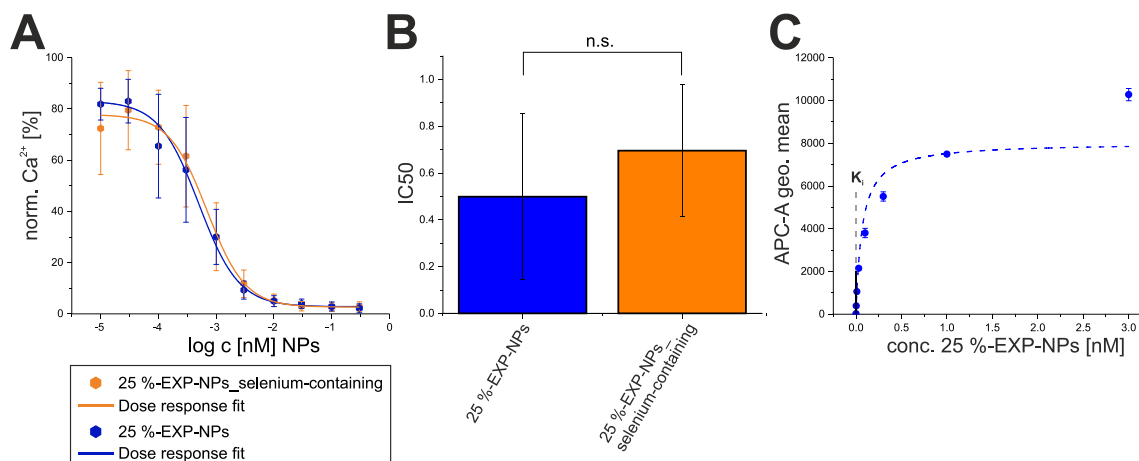


Figure 6 Determination of the K_i values for both types of model nanoparticles. (A) Binding curves of 25 %-EXP-NPs and 25 %-EXP-NPs_selenium-containing derived from Fura-2 AM based Ca^{2+} mobilization assay. Binding of the agonist causes an increase of the cytosolic Ca^{2+} concentration by inducing the G protein-coupled receptor (GPCR) activation cascade,^[45,46] as indicated by the fluorescent Ca^{2+} chelator Fura-2 AM. Results represented as averaged data from five independent experiments ($N = 5$, $n = 3$). (B) IC_{50} values of both nanoparticle types calculated from the averaged data. Derived IC_{50} values were compared via Welch's t-test ($Pr(>t) = 0.1039$). Levels of statistical significance are indicated as n.s. – not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. (C) The binding curve of CY-5-labeled 25 %-EXP-NPs was recorded on rMCs via flow cytometry measurements. Data represented as mean $\pm$ SD ($n = 3$) and the binding curve was plotted using Michaelis Menten fit. The K_i value of the nanoparticles calculated using Cheng-Prusoff transformation was fitted into the plot as an intercept.

To determine the number of nanoparticles bound to the cell surface (N_{NP}), binding data on rMCs were recorded by flow cytometry as a first approximation (Figure 6C). The derived binding curve showed saturation behavior, i.e. the saturability of the cell surface with nanoparticles. When the K_i value of the nanoparticles was fitted to the graph, the curve was intersected in the slope before the saturation plateau was reached, indicating a quantitative binding of the nanoparticles in this concentration. To further evaluate the nanoparticle binding quantitatively, the concentration of selenium-tagged

nanoparticles adhering to the cell matrix (N_{NP}) at their K_i value concentration was measured using ICP-MS. The selenium content of the cell samples was quantified to be $36 \pm 7 \mu\text{g L}^{-1}$ (mean $\pm$ SD, from three experiments). This corresponds to a nanoparticle concentration of $0.066 \pm 0.015 \text{ pM}$ associated with the cell surface (Figure S8).

The average number of binding ligands per nanoparticle (N_{bound}) could be determined by inserting the variables obtained from the successive experiments into equation 6. Initially assuming quantitative binding of the nanoparticles to the cell surface, as can be derived from flow cytometry data, N_{bound} was calculated to 85 ligands. Correcting this number by the data obtained from the ICP-MS measurements, N_{bound} increased to 251 ligands. Taking these results together led to a range of 85 to 251 binding ligands per nanoparticle: [85; 251] (Figure 7). It can therefore be summarized that on average a maximum of 251 ligands per nanoparticle bound to targeted receptors on the cell surface. N_{bound} was related to the determined mean ligand valence to obtain the fraction and density of binding ligands (Figure 7). Both quantities varied greatly depending on the method used to estimate the mean ligand valence (Table S2). In relation to the maximum of 251 binding ligands, a fraction of 3.7 % to 65.1 % binding ligands could be determined, corresponding to a density of 0.8 to 5.3 binding ligands per 100 nm^2 surface area (data represents the geometric mean).

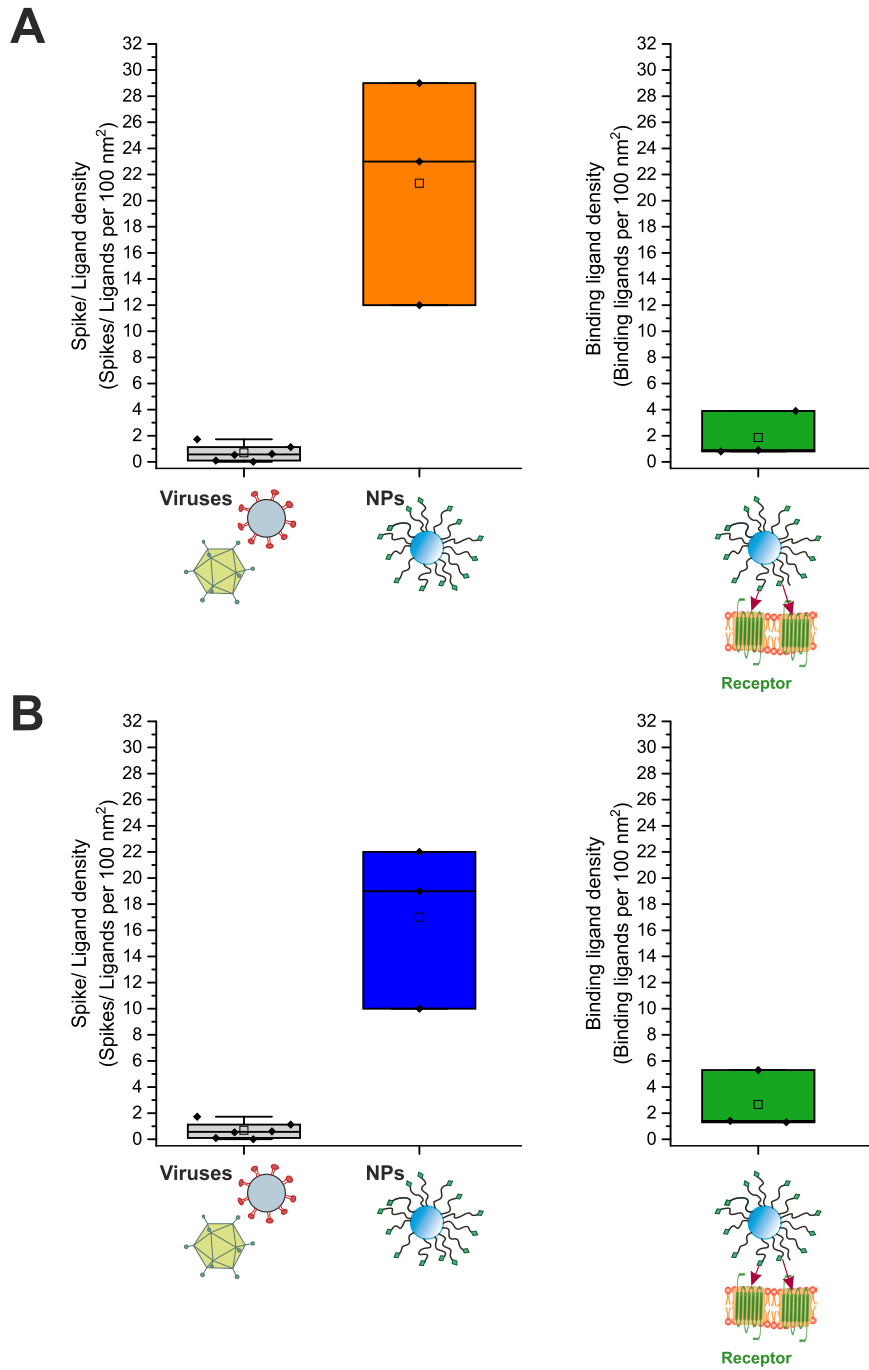


Figure 7 Spike and ligand density on viruses and nanoparticles. (A) Ligands per 100 nm² nanoparticle surface area in direct comparison to viral role models and binding ligands per 100 nm² nanoparticle surface area for selenium-tagged and (B) unlabeled nanoparticles. For the underlying evaluation, the DLS data were analyzed as intensity percent. The values for the viruses were taken from the publication by Alkilany et al..^[4]

Considerations on Nanoparticle Binding in Clathrin Coated Pits (CCPs)

We have recently demonstrated that the presence or absence of clathrin coated pits (CCPs), which play a crucial role in the receptor-mediated endocytosis of both, viruses, and nanoparticles,^[47,48] is an influential factor in the avidity of multivalent nanoparticles.^[13] In terms of their shape and diameter, CCPs align well with the geometry of nanoparticles (spherical geometry, $d < 100$ nm).^[49] They thus increase the number of possible ligand-receptor interactions compared to the flat membrane.^[13] Consequently, we aimed to assess whether our current results could be reconciled with this, and nanoparticles could bind to 251 receptors located in CCPs.

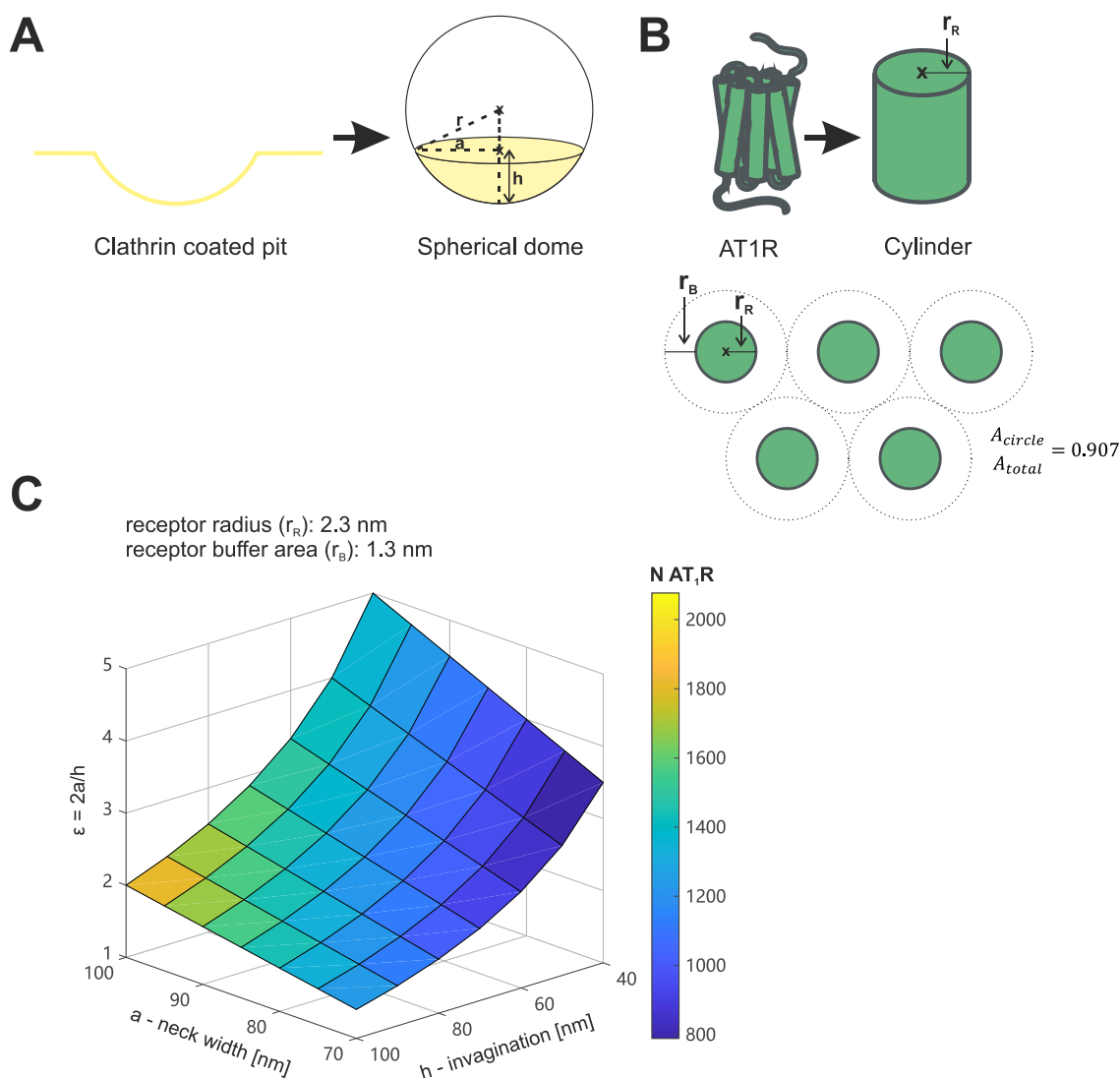


Figure 8 Illustration of the considerations regarding the geometry of AT1R and CCPs, and the calculation of the number of AT1Rs located in CCPs. (A) Simplified model of clathrin coated pits as a spherical dome, with r referring to the radius of the sphere, a indicating the radius of the base circle of the spherical segment and h being the height of the spherical dome. (B) Description of the geometry of the AT1R using the simplified model of a cylinder with the radius r_R and illustration of the correction factor for the closest packing of circles (0.907). (C) Color coded plot of the AT1R number in a CCP depending on a , h and ε , with ε accommodating the stage of development of the CCPs depending on a and h , which are described in Figure 8A.

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To the best of our knowledge, there is no data available in the literature regarding the number or density of AT1R in isolated CCPs. Therefore, theoretical considerations were used to derive an estimate based on available data about CCP geometry and AT1R dimensions (Figure 8). The geometry of CCPs was simplified to a spherical dome with different diameters (d) and depths (h) depending on their stage of development.^[49,50] The geometry of the AT1R was described by the facilitated model of a cylinder. In a previous study, we have calculated the radius (r_R) of a single AT1R to be 2.3 nm;^[35] however, this does not provide a direct indicator of the space occupied by a receptor on the cell membrane. Aldossary et al.^[51] have recently published a study on the distribution and clustering of AT1R on rat pheochromocytoma cells (PC12 cells). These data serve as at least a good approximation for the rat mesangial cells we used due to the origin from the same species. They demonstrated a cluster density of $2.2 \cdot 10^4$ localizations per μm^2 in AT1R clusters on the cell surface (data represents the mode). Based on their data, we calculated the minimum area occupied per receptor to be 3.6 nm^2 . The number of AT1R per CCP ($N_{\text{AT1R/CCP}}$) could be estimated by dividing the surface area of the CCP by the membrane area reserved for a single AT1R. *(Please refer to the supplementary information for the details of the calculation and the assumptions made.)*

Data of the geometric features of CCPs in their early stage of development, as they are expected to occur during their initial contact time with nanoparticles, were taken from a study published by Kozik et al.^[49] and plotted as a three-dimensional surface. The obtained plot showed the calculated $N_{\text{AT1R/CCP}}$ color coded (Figure 8). $N_{\text{AT1R/CCP}}$ was estimated on a minimum of 800 and a maximum of 2000 receptors depending on the geometric feature of the CCP under consideration and assuming the densest packing of the receptors. It is therefore mathematically plausible that nanoparticles bind with an average of 251 ligands to the receptors in CCPs. However, these figures must be scrutinized against the biological context.

Discussion and Conclusion

In the present study, we used engineered polymer nanoparticles as a model system to quantify the average number of ligands per nanoparticle that bind to target cell receptors on the cell surface, finding that the maximum number of binding ligands was 251. A look at the literature reveals that very few studies have addressed this question so far,^[6,14] which were frequently based on antibody-functionalized model nanoparticle systems. The computational study by Farokhirad et al.^[12] provides at least a benchmark for our work as they analyzed a rigid-tethered nanoparticle model. They calculated the number of binding ligands as a function of the excess area of the (cell) membrane in a range of 45 to 73 binding ligands. These values are rather comparable to the minimum of 85 binding ligands that we calculated assuming quantitative binding of the nanoparticles to the cells surface. However, it is important to mention that the nanoparticles in our study were investigated within a specific biological context, namely in their interaction with AT1 receptor expressing rMCs. It has been shown that GPCRs are often not randomly distributed on the cell membrane, but rather compartmentalized into functional domains.^[52] This also applies to AT1Rs, which are organized into microdomains as clusters on various types of kidney cells.^[51,53] Upon activation by an agonist, such as angiotensin-II, a partial restructuring of the receptor distribution can be observed, whereas stimulation with antagonists does not induce this restructuring.^[53] It can therefore be assumed that the binding of EXP3174-functionalized nanoparticles has no effect on the distribution of AT1Rs on the cell surface. For PC12 cells, Aldossary et al.^[51] described the organization of AT1Rs in clusters, with an average of 252 ± 15 receptors per cluster (with a range of 10-1041 receptors and more than 50 % of the clusters containing fewer than 100 receptors). The cluster size makes it plausible that up to 251 receptor bonds per nanoparticle could form, given that the membrane undergoes bending. Since we were able to demonstrate in a previous study that the presence of CCPs in the membrane increases the avidity of nanoparticles,^[13] the question arises as to whether receptor clusters on the membrane are associated with CCPs. Given the high technical complexity, limited data is available on this topic. Nevertheless, the findings of Liu et al.^[54] indicate at least for transferrin receptors a positive correlation between receptor clustering and the formation of CCPs. Comparing the mean area of a AT1R cluster on PC12 cells of about $9.8 \pm 0.6 \cdot 10^{-3} \mu\text{m}^2$ (with more than 50 % of the clusters having an area of less than $3.7 \cdot 10^{-3} \mu\text{m}^2$) with the average surface area of a typical early-stage CCP of about $6.5 \cdot 10^{-3} \mu\text{m}^2$ (assuming a diameter of 100 nm),^[36] it is quite conceivable that some of the receptor clusters are located in the region of preexisting CCPs or respectively that CCPs could form in the region of receptor clusters. Although the association with CCPs remains to be confirmed experimentally, considering the biological context and the presence of AT1R clusters clearly supports the data obtained in this study.

The comparison with viral role models impressively showed that the nanoparticles under consideration exhibited a target cell avidity comparable to that of viruses, for which K_D values in a broad range from mM to pM have been reported.^[55–59] However, the nanoparticles possessed a significantly higher quantity of ligands, as their ligand density exceeded that of viruses by at least a

factor of 10.^[4] The high ligand density on the surface of nanoparticles is certainly a factor that weakens their cell selectivity. This is strongly underlined by the fact that only a maximum of 5.3 ligands per 100 nm² nanoparticle surface were actually involved in specific ligand-receptor interactions. The density of binding ligand-bearing chains closely approximates to the total spike density of viruses. However, a direct comparison with viruses in terms of the average number of binding ligands is challenging, as it heavily depends on the virus type. It has been shown that a low spike density is often sufficient for a virus particle to be infectious, with saturation effects on infectivity observed as the number of spikes increases.^[4,60] The binding events of viruses can be directed by controlled lateral movements on the cell surface,^[61] surface patterns that negatively regulate the number of binding spikes,^[62] the specific steric alignment of spikes in the direction of the target^[63] and the formation of specific binding motifs.^[64] For instance, the formation of an “entry claw” consisting of five to seven spike receptor rods between SIV/HIV-1 and T Cells has been documented in the literature.^[64] In other words, mechanisms associated with the movement of spikes can lead to a temporary punctual clustering of the spikes on the surface of viruses. Given that nanoparticles cannot make use of such mechanisms and considering that not all ligands are likely to possess a favorable steric orientation, it is plausible that a more extensive functionalization with ligands is required to achieve comparable avidities. Surface-sensitive techniques such as atomic force microscopy (AFM) or surface plasmon resonance spectroscopy (SPR) have recently also been used to determine the number of binding functionalities of viruses.^[62,65–67] Delguste et al.^[62] functionalized AFM cantilevers via PEG-linkers with virions to characterize the multivalent binding of herpesviruses to living cells. The use of AFM as a state-of-the-art surface-sensitive technique can also be considered promising for the quantification of binding events between nanoparticles and cell surfaces. However, there are only few studies that have used AFM in this context.^[14,68] We posit that AFM offers the advantage of enabling the direct and real-time measurement of surface interactions. Conversely, it has to be considered that the functionalization of AFM cantilever tips with nanoparticles creates highly artificial systems. It restricts the degrees of freedom of movement of the attached particles and consequently influences the binding behavior to the cell surface. When moving the functionalized cantilever tip over the cell surface, the binding of the attached particle to the cell surface can only be measured as a local event. This contradicts our understanding of nanoparticle binding as a global event,^[5] which also depends on cooperative effects between nanoparticles^[69] and the competition for a limited number of surface receptors.^[5] Regardless on the method employed to investigate the question, it must be considered that in a serum-containing environment, proteins and lipids adsorb to the surface of nanoparticles and thus influence their interaction with the cell surface.^[70–72] The identity of the nanoparticles is significantly affected as a result.^[73] In case of the nanoparticles under consideration, it can therefore not necessarily be assumed that all ligands on their surface were “visible” and thus available for binding. However, since their avidity was determined in a competition assay, it can be concluded that at least a substantial fraction of the ligands on the nanoparticles surface was available for specific interactions with the receptors.

The implications of our findings go further than a more precise understanding of the interactions between nanoparticles and cells at the level of individual receptors. They contribute directly to tailored

development approaches for the design of selective nanoparticles. The aim of a competitive multivalent nanoparticle design must be to adjust the ligand valency so that an enthalpic gain and therefore the threshold for successful binding is reached.^[16] Conversely, overloading nanoparticles with ligands beyond the optimum has detrimental effects on the target selectivity and uptake kinetics.^[6,17,21,74] As target and off-target cells often express the same receptors only to different degrees, it is important that distinguishable binding levels and thus superselectivity for the nanoparticles are achieved.^[6] Adjusting the number of ligands to the number actually required for anchoring to their target cells, would therefore help to optimize nanoparticle uptake rates and kinetics as well as their target specificity, transforming them into more successful drug-delivery or diagnostic systems. Despite the significant role of the spike density emerging from the viral infection process, this design parameter continues to be largely neglected in current nanoparticle research.^[15] This could also be due to the fact that simply reducing the ligand number on the nanoparticle surface is likely an oversimplification, as ligand-bearing polymer chains, unlike viral spikes, are not spatially mobile on the surface and therefore cannot locally condense. Our research suggests that the key may therefore lie in focusing on the structures of the biological target (with particular attention to receptor expression patterns) and tailoring the nanoparticle design by adjusting on the one hand the ligand number and on the other their arrangement. E.g. the functionalization of nanoparticles with ligands in form of clusters could be a promising concept as already described by Poon et al..^[75] By presenting an experimental approach to quantify the number of binding ligands that copes with the complexity of the system, we lay the foundation for such design strategies, which could be the next leap in the development process of active targeting concepts.

Material and Methods

Materials

All materials and reagents were purchased from Sigma Aldrich (Taufkirchen, Germany or St. Louis, USA) unless otherwise stated. Dulbecco's phosphate-buffered saline (DPBS) was acquired from Gibco™ (Life Technologies, Paisly, UK), and Leibovitz's L-15 medium was purchased as medium without phenol red from Gibco™ (Life Technologies, New York, USA). The ultrapure water used for the experiments was treated with a Milli-Q EQ 7000 system (Merck, Darmstadt, Germany) equipped with Milli-Q Biopak filter (Merck, Darmstadt, Germany) and freshly taken every day. It is referred to as Milli-Q water in the following.

Cell Culture

Rat mesangial cells (rMCs) were kindly gifted by Prof. Armin Kurtz (Institute of Physiology, University of Regensburg, Regensburg, Germany). The cell line was chosen as a model cell line for the cell surface interaction studies due to the stable expression of the target receptor, angiotensin-II type 1 receptor (AT1R), as previously shown by Maslanka et al..^[1] Cells of passage number 78 were used for all experiments directly relevant to the calculated result, to ensure a constant receptor number in the critical experiments. The cells were cultured in RPMI 1640 medium containing 10 % fetal bovine serum (FBS) (South America Origin, Pan-Biotech, Aidenbach, Germany, Lot-No.: P201004) supplemented with insulin-transferrin-selenium (ITS) (Gibco, Life Technologies, New York, USA) and 100 nM hydrocortisone in a water saturated, carbon dioxide enriched (5 % CO₂) atmosphere at 37 °C.

Cell Counting

Cell counting with Neubauer Improved Counting Chamber (Marienfeld, Lauda-Königshofen, Germany) was performed as a double sampling in both counting areas of the chamber. The cells were counted in 20 group squares in the middle square on each side of the chamber.

Losartan Carboxylic Acid (EXP3174) Synthesis and Characterization

Losartan carboxylic acid (EXP3174) was obtained by oxidation of losartan with potassium permanganate as previously described by our group.^[76] Therefore, losartan potassium (Santa Cruz Biotechnology, Heidelberg, Germany) (2.0 g, 4.4 mmol) was diluted in 1 M sodium hydroxide solution (NaOH) (50 mL) and cooled down to 0 °C. An ice-cold, aqueous solution of potassium permanganate (Merck, Heidelberg, Germany) (1.58 g, 10.0 mmol in 50 mL) was added dropwise. The reaction mixture was stirred for 4.5 h at room temperature (RT). The product was then precipitated by adjusting the pH to acidic conditions with 1 M hydrochloric acid (HCl). The solid was isolated by

centrifugation (3000 g, 4 °C, 15 min) and washed twice with ice-cold water. The product was isolated by resuspending the solid in water and adjusting the pH to moderately alkaline conditions with 1 M NaOH. Remaining precipitated manganese dioxide was removed by centrifugation (3000 g, 4 °C, 15 min). The product was precipitated from the supernatant by acidification with 1 M HCl and then extracted with ethyl acetate in a separating funnel. The solvent was eliminated through rotary evaporation, the product was dissolved in ethanol, precipitated in an excess of water and then freeze-dried (Alpha 2-4 LSCplus, Christ, Osterode am Harz, Germany) to obtain a dry powder (reaction yield: 50 %). Identity and purity of EXP3174 were verified by ^1H -NMR and high-resolution mass spectrometry analyses. The ^1H -NMR spectrum was recorded on a Bruker Avance III HD 400 operating at 400 MHz. The product was therefore diluted in deuterated dimethyl sulphoxide (DMSO- d_6). Topspin 4.1.4 software was used to evaluate the ^1H -NMR spectrum. Mass spectrometry analyses were performed on a Q-TOF 6540 ultrahigh definition (Agilent Technologies, Waldbronn, Germany) using an electrospray ionization source (ESI). (Figure S4)

Block Copolymer Synthesis and Characterization

$\text{COOH-PEG}_{5\text{k}}\text{-PLA}_{10\text{k}}$, $t\text{Boc-NH-PEG}_{5\text{k}}\text{-PLA}_{10\text{k}}$, and $\text{MeO-PEG}_{5\text{k}}\text{-PLA}_{10\text{k}}$ block copolymers were synthesized via ring-opening polymerization of the cyclic lactone 3,6-dimethyl-1,4-dioxane-2,5-dione with the organocatalyst 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) at RT as previously described by our group.^[77] $\text{COOH-PEG}_{5\text{k}}\text{-OH}$ and $t\text{Boc-NH-PEG}_{5\text{k}}\text{-OH}$ (Jenkem Technology, Plano, USA, $M_n 5000 \pm 500 \text{ g mol}^{-1}$, as stated on certificate of analysis) and $\text{MeO-PEG}_{5\text{k}}\text{-OH}$ ($M_n 5000 \text{ g mol}^{-1}$) were used as macroinitiators for the reaction.^[78,79] In brief, polyethylene glycol (PEG) macroinitiators (800 mg, 160 μmol) were reacted with 3,6-dimethyl-1,4-dioxane-2,5-dione (1.60 g, 11.1 mmol) and DBU (84.2 μL , 553 μmol) in dichloromethane (DCM) as solvent at RT. After 1 h the reaction was quenched with benzoic acid in excess (0.34 g, 2.8 mmol). The resulting block copolymers were precipitated in ice-cold diethyl ether, filtered, and dried under the nitrogen flow (reaction yield, $\text{COOH-PEG}_{5\text{k}}\text{-PLA}_{10\text{k}}$: 62 %; reaction yield, $\text{MeO-PEG}_{5\text{k}}\text{-PLA}_{10\text{k}}$: 34 %). In case of *tert*-butyloxycarbonyl (*t*Boc)-protected block copolymers, the protecting group was subsequently removed by addition of trifluoroacetic acid (TFA) (reaction yield, $\text{NH}_2\text{-PEG}_{5\text{k}}\text{-PLA}_{10\text{k}}$: 49 %). Identity and purity of the block copolymers were verified by ^1H -NMR spectra recorded on a Bruker Avance III HD 400 operating at 400 MHz; the block copolymers were dissolved in deuterated chloroform (CDCl_3).^[80] Topspin 4.1.4 software was used to evaluate the ^1H -NMR spectra, exemplary shown for $\text{PLA}_{10\text{k}}\text{-PEG}_{5\text{k}}\text{-NH}_2$ (Figure S2). Furthermore, the molar mass of the synthesized polymers was determined from the ^1H -NMR spectra as previously described by our group^[13] (equation 9):

$$M_{\text{PLA-polymer}} = \frac{(I_{\text{PLA,CH}_3} + I_{\text{PLA,CH}}) \cdot M_{\text{PLA-unit}}}{I_{\text{PEG}} \cdot M_{\text{PEG-unit}}} \cdot M_{\text{PEG-polymer}}$$

eq. 9

The number-average molar mass of the PLA polymer ($M_{\text{PLA-polymer}}$) was calculated from that of the PEG polymer ($M_{\text{PEG-polymer}}$) by relating the integrals of their signals in the NMR spectrum: $I_{\text{PLA-CH}_3}$

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corresponds to the lactic acid ($-\text{CH}_3$) integral, $I_{\text{PLA-CH}}$ corresponds to the lactic acid ($-\text{CH}-$) integral, and I_{PEG} is the PEG ($-\text{OCH}_2\text{-CH}_2-$) integral (Figure S2). $M_{\text{PLA-unit}}$ and $M_{\text{PEG-unit}}$ are the molar masses of PLA and PEG monomers.

EXP3174 Coupling

For EXP3174-PEG_{5k}-PLA_{10k}, the deprotected amino-terminated block copolymer was functionalized with EXP3174 using EDC/NHS coupling chemistry. The ligand was coupled via its carboxylic acid functionality to the polymer. To activate the carboxylic function, EXP3174 (80 mg, 0.18 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (100 mg, 520 μmol) and *N*-hydroxysuccinimide (NHS) (30 mg, 0.26 mmol) were dissolved in 3000 μL dry *N,N*-dimethylformamide (DMF). Subsequently, a solution of NH_2 -PEG_{5k}-PLA_{10k} (800 mg, 53.3 μmol) and *N,N*-diisopropylethylamine (DIPEA) (178.5 μL , 1024 μmol) in 1000 μL DMF was added dropwise to the ligand solution. The reaction mixture was stirred for 24 h at RT. For purification, the functionalized polymer was precipitated in a mixture of methanol and diethyl ether (1:15, v/v) and the resulting product was dialyzed (using SpectraPor 6-8 kDa molecular weight cut-off, RC membrane, Spectrum Laboratories, Inc, Los Angeles County, USA) against ethanol, 100 mM borate buffer (pH 8.5) and Milli-Q water (1:1:8, v/v) for 24 h followed by Milli-Q water for 12 h. The product was freeze-dried (Alpha 2-4 LSCplus, Christ, Osterode am Harz, Germany) to obtain a dry powder (reaction yield: 95 %). The coupling efficiency was determined by detecting free, unreacted amino groups via Fluram assay. (Figure S5)

Iodine Assay and Fluram Assay

Iodine Assay: The PEG amount was quantified via iodine assay adapted from Childs^[81] as previously published by our group.^[77] In brief, poly(ethylene glycole) monomethyl ether with an average molecular weight of 5000 g mol⁻¹ (mPEG5k) was used as calibration standard in aqueous solutions (0–20 $\mu\text{g mL}^{-1}$). 140 μL of standard dilutions and polymer micelles as samples were pipetted into a 96-well plate and incubated for 10 min under light exclusion with 60 μL of a solution composed of 5 % barium chloride (m/V) (Merck, Darmstadt, Germany) in 1 M HCl (3 mL, 2 equiv.) and 0.05 M iodine solution (VWR, Lueven, Belgium) (1.5 mL, 1 equiv.). The absorbance was measured at λ 535 nm on a FLUOstar Omega plate-reader (BMG Labtech, Ortenberg, Germany). (Figure S5)

Fluram Assay: The detection of free amino groups was carried out using the Fluram assay adapted from Udenfriend et al..^[82] Micelles of amino-terminated polymer served as positive control and micelles of methoxy-terminated polymer as negative control. Positive and negative control were mixed at specific concentration ratios (amino- to methoxy-terminated, 100:0 / 80:20 / 60:40 / 40:60 / 20:80 / 0:100) to record a calibration line while maintaining the polymeric matrix. Briefly, 10 μL of the respective test solution (sample, positive or negative control) were added to a white 96-well plate (Greiner Bio One, Frickenhausen, Germany) with 170 μL of borate buffer (pH 8.5, 50 mM) and shaken (150 rpm) for 5 min. Afterwards, 20 μL of a solution of 4'-phenylspiro[2-benzofuran-3,2'-

furan]-1,3'-dione, fluorescamine (fluram) in acetone (0.3 mg mL^{-1}) were added and incubated together with the test solutions at RT for 15 min under shaking (150 rpm). The fluorescence was measured on a FLUOstar Omega plate-reader (BMG Labtech, Ortenberg, Germany) with an excitation wavelength of λ 380 nm. The emission was recorded on a wavelength of λ 485 nm. The samples were evaluated against the calibration line. (Figure S5)

Fluorescence Labeling of PLGA with CY-5

Poly(D,L-lactide-co-glycolide) (PLGA) (Resomer RG 502, carboxylic acid-terminated, M_w 7000-17000 g mol^{-1} , Sigma-Aldrich, St. Louis, USA) was fluorescently labeled by covalent linkage with cyanine-5-amine (Lumiprobe GmbH (Europe), Hannover, Germany) as previously described by our group (Figure S3).^[77] In brief, carboxylic acid-terminated PLGA (0.92 mg, 77 μmol) was dissolved together with cyanine-5-amine (CY-5) (5.0 mg, 7.7 μmol) in dry DMF. The carboxylic acid group was activated by the addition of (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (58 mg, 0.15 mmol) and DIPEA (53.5 μL , 306 μmol). The reaction mixture was stirred overnight at RT under light protection. To remove unreacted free dye, the polymer was precipitated several times in ice-cold diethyl ether until the supernatant was no longer blue. The product was dried under the nitrogen flow (reaction yield: 93 %) and characterized by FTIR spectroscopy. FTIR spectra were recorded on an Agilent Technologies Cary 630 FTIR (Agilent, Santa Clara, USA) with 32 scans, 8 cm^{-1} resolution (Figure S3). Furthermore, absorbance spectra of the PLGA and CY-5-PLGA in acetonitrile (4 mg mL^{-1}) (ACN) were recorded (Figure S3).

Nanoparticle Preparation

Core-shell nanoparticles were obtained by nanoprecipitation.^[77,83] For this purpose, the synthesized block-copolymers and poly(D,L-lactide-co-glycolide) (PLGA) (Resomer RG 502, ester-terminated, M_w 7000-17000 g mol^{-1} , Sigma-Aldrich, St. Louis, USA) were dissolved in ACN to a concentration of 10 mg mL^{-1} . The ratio of PLGA core to the block-copolymer was 3:7 (m/m). To achieve the desired degree of surface functionalization of the nanoparticles, the appropriate amount of EXP3174-terminated polymer within the block-copolymers was utilized. The organic phase was added dropwise into the 10-fold excess of stirring (930 rpm) aqueous phase (10 % DPBS (v/v) in Milli-Q water) and stirred for 3 h to remove the ACN. Subsequently, the volume was concentrated by centrifugation at 3000 g for 15 min using Microseps Advance 30K centrifugal filters (molecular weight cut-off 30 kDa, Pall Corporation, New York, USA).

Preparation of Selenium-Containing Nanoparticles

Selenium-containing core-shell nanoparticles were prepared by modifying the nanoprecipitation method described above. Selenium was generated in situ by reducing sodium selenite (80.9 μL of a 0.1 M aqueous solution) with ascorbic acid (80.9 μL of a 0.2 M aqueous solution) (Caesar & Loretz

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GmbH, Hilden, Germany) in 4.0 mL of the aqueous phase (10 % DPBS (v/v) in Milli-Q water), as already described in the literature.^[21] One minute after the start of the redox reaction, 0.4 mL of the organic phase containing the polymer were added dropwise into the vigorously stirring aqueous phase. The mixture was stirred for 3 h to evaporate the ACN and to embed the elemental selenium in the polymer matrix and thus stabilize it. The selenium-containing nanoparticles were concentrated by centrifugation at 1500 g for 15 min using Microseps Advance 30K centrifugal filters (molecular weight cut-off 30 kDa, Pall Corporation, New York, USA).

Nanoparticle Characterization

The nanoparticles were characterized as previously published by our group^[77]: Dynamic light scattering (DLS)-based measurements (particle size distribution, polydispersity index, zeta potential) were carried out with a Malvern Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany) equipped with a λ 633 nm He-Ne laser operating at an angle of 173°. The software used for data collecting and processing was Zetasizer software version 7.12 (Malvern Instruments, UK). To determine the size distribution, the concentrated samples were diluted 1:20 with 10 % DPBS in Milli-Q water (10 % DPBS (v/v)). 90 μ L sample volume were measured in micro-UV-cuvettes (Carl Roth, Karlsruhe, Germany) at a controlled temperature of 25 °C (120 s equilibration time). Three repetitions with 11 runs of 10 sec duration were performed for each sample. The data was analyzed with the software-integrated analysis model for “general purposes (normal resolution)”. For zeta potential measurements the samples were diluted 1:200 with 10 % PBS in Milli-Q water. Samples were measured in folded capillary zeta cells (Malvern, Herrenberg, Germany) at 25 °C. Three repetitions with at least 60 runs were performed for each sample. The data was analyzed by “auto mode”. The result of the measurement is a discretized particle size distribution.

Furthermore, the average size (hydrodynamic diameter), size distribution and concentration [particles mL⁻¹] of the nanoparticles were determined using nanoparticle tracking analysis (NanoSight NS300, Malvern, Worcestershire, UK). The device was equipped with an O-Ring top-plate and the data was analyzed with NanoSight NTA 3.4 software (NTA Malvern Panalytical). Before measurement, all samples were diluted with Milli-Q water to achieve a good particle per frame value (20-100 particles/frame). The samples were applied by manual injection with sterile syringes. Each sample was measured in three cycles of 60 s duration (screen gain: 1.0; camera level: 16) at RT. The detection threshold was set at 3.

Fura-2 AM-Based Ca²⁺ Mobilization Assay

The calcium influx into the cytosol after AT1R activation was measured using Fura-2 AM-based ratiometric Ca²⁺ assay as previously described in literature.^[13,40,41] Therefore, rMCs were harvested, centrifuged (0.2 g, 5 min) and carefully resuspended in Leibovitz's-based Fura-2 AM loading medium, composed of 25 μ g Fura-2 AM (Invitrogen, Thermo Scientific, Oregon, USA) pre-resolved in 25 μ L cell culture grade DMSO, 10 μ L 20 % Pluronic F-127 and 2.5 mM probenecid in Leibovitz's

L-15 medium. After 1 h of incubation on the lab shaker (50 rpm) under light protection, the cells were centrifuged again (0.2 g, 5 min) for discarding the Fura-2 AM loading medium and resuspended in Leibovitz's measurement buffer containing 2.5 mM probenecid.

To determine the equilibrium dissociation constant (K_D) of Ang-II, the cell number was adjusted to $1.0 \cdot 10^6$ cells mL⁻¹ via Neubauer Improved Counting Chamber (Marienfeld, Lauda-Königshofen, Germany). 20 μ L of a ten-fold over-concentrated serial dilution of Lys-Ang-II was pipetted into a white 96-well plate (Greiner Bio One, Frickenhausen, Germany). 180 μ L of the cell suspension were added via FLUOstar Omega pump system set to a speed of 100 μ L s⁻¹ (Figure S11).

To determine the half maximal inhibitory concentration (IC_{50}) of EXP3174-functionalized NPs, the cell number was adjusted to $2.0 \cdot 10^6$ cells mL⁻¹ via Neubauer Improved Counting Chamber (Germany, Marienfeld). For the experiment 20 μ L of a ten-fold over-concentrated serial dilution of NPs were incubated with 90 μ L cell suspension in a white 96-well plate for 30 min at RT under moderate shaking (50 rpm). After the incubation period, 90 μ L of a 300 nM Lys-Ang-II solution (sequence: NH₂-KDRVYIHPF-COOH, M_w 1173.6 g mol⁻¹; GenScript, Rijswijk, Netherlands) were added via the FLUOstar Omega pump system to activate the non-inhibited part of the AT1R and induce calcium influx (Figure S11). For optimization of assay sensitivity and reproducibility it is recommended to use agonists at the concentration of their EC_{80} value when antagonism is assessed in competition assays.^[84] The EC_{80} value was determined in advance for the free agonist Lys-Ang-II to 135 nM, also by measuring the Ca²⁺ influx.^[41]

In both experimental settings, the fluorescence intensity was measured ratiometrically on a FLUOstar Omega plate-reader (BMG Labtech, Ortenberg, Germany) exciting the samples alternately at λ 340/20 nm and λ 380/20 nm every 1.5 s. The emitted light was recorded at λ 510/20 nm for 30 s. To calculate the intracellular Ca²⁺ levels, the highest measured ratio of each sample was used and the value was entered into the Grynkiewicz-equation.^[40] The maximum ratio (R_{max}), required in the equation for calibration, was determined by lysing the cells with 20 μ L of 1 % Triton® X-100 in PBS. Similarly, the minimum ratio (R_{min}) was obtained by incubating the cells with 20 μ L of 1 % Triton® X-100 in PBS supplemented with 45 mM of ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), a strong calcium chelating agent, and 0.5 M NaOH. The K_D value for the Fura-2-calcium complex was taken from the literature with a value of 225 nM.^[40] The data was plotted and analyzed with Origin 2022b software (OriginLab Corporation, Northampton, Ma, USA).

CLSM Analysis

Confocal laser scanning microscopy images were recorded on a Zeiss LSM 710 (Carl Zeiss Microscopy GmbH, Jena, Germany) (Figure S6 and S7). The images were processed and analyzed using ZEN 3.5 blue edition software. In preparation for image acquisition, 20,000 rMCs/well were seeded in an Ibidi 8-well chamber (Ibidi, Grafelfing, Germany) and incubated for 24 h at 37 °C (5 % CO₂). Fluorescent nanoparticles were manufactured according to the procedure described above using CY-5-labeled PLGA. The concentration was adjusted to 3 nM in Leibovitz's L-15

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medium. Before sample application, the cell medium was removed, and the cells were washed twice with pre-warmed DPBS. Next, the cells were incubated with 200 μ L of the nanoparticle dispersion for 30 min at 37 °C (5 % CO₂) or at RT. After the incubation period, the nanoparticle excess was aspirated, and the cells were washed with DPBS. For fixation, the cells were washed with pre-warmed DPBS once and then incubated with 4 % paraformaldehyde (PFA) (Merck, Darmstadt, Germany) solution for 10 min at RT. The nuclei were stained by incubating the cells with 4',6-diamidino-2-phenylindole (DAPI) staining solution (1 μ g mL⁻¹ in PBS) for 10 min at RT. After another washing step with DPBS, the cell membrane was stained with 10 μ M organic 3,3'-dioctadecyloxacarbocyanine (DiO) staining solution in DPBS for 15 min at RT. Finally, the samples were stored on DPBS in the dark at 2-8 °C.

Flow Cytometry

RMCs were seeded in TC-treated 24-well plates (Costar, Corning Incorporated, New York, USA) at a density of 60,000 cells/well and cultivated for 24 h (37 °C, 5 % CO₂) before proceeding for the fluorescence-activated cell sorting (FACS) analysis. On the day of experiment, the cells were at least 90 % confluent. To record the nanoparticle binding curve, serial dilutions (3 nM to 3 pM) of fluorescent EXP3174-functionalized nanoparticles were prepared in Leibovitz's L-15 medium. The cells were set up for the experiments by discarding the RPMI 1640 medium. They were then incubated with 200 μ L of each nanoparticle dilution (3 replicates) under gentle shaking (50 rpm) for 30 min (37 °C, 5 % CO₂). After the incubation period, the nanoparticle dilutions were discarded, and the cells were washed once on-plate with 500 μ L DPBS. For harvesting, 300 μ L of trypsin (PAN-Biotech, Aidenbach, Germany) were added and the cells were incubated for 2 min (37 °C, 5 % CO₂). After cell detachment, 500 μ L of ice-cold RPMI 1640 were added to quench trypsinization. The samples were transferred to 2.0 ml Eppendorf-Tubes and centrifuged (0.2 g, 5 min, 4 °C). The supernatant was discarded, and cells were washed twice with ice-cold DPBS under centrifugation. The cell pellets obtained after the last washing step were resuspended in 300 μ L ice-cold DPBS and kept on ice until measurement. FACS analyses were performed on a FACS Canto II (Becton Dickinson, Franklin Lakes, USA). The fluorescence of the CY-5-labeled nanoparticles was excited at λ 633 nm and the emission was recorded using a λ 661/16 nm bandpass filter. The data was analyzed and plotted using Flowing software (v2.5.1, Cell Imaging and Cytometry Core, Turku Bioscience Centre, Turku, Finland, with the support of Biocenter Finland). Further evaluations were carried out based on the determined geometric mean of the fluorescence intensity.

Transmission Electron Microscopy

For sample preparation, 9 nm carbon film coated 3.5 nm Cu TEM grids (400 mesh) (Plano GmbH, Wetzlar, Germany) were hydrophilized in the oxygen plasma. 5 μ L of the respective nanoparticle dispersion were applied to the grids for 5 s and subsequently for 1 min. Afterwards, the excess samples were removed carefully with a filter paper, and the copper-grids were washed with Milli-Q

water and exposed for 30 s to an aqueous dilution of uranyl acetate (1 %). A stack of images at different positions was recorded on a JEM-2100F Field emission electron microscope (Jeol, Freising, Germany) at 20,000-fold magnification using the SerialEM software. The size of 500 nanoparticles of each batch was analyzed using Fiji software.^[85] The size distribution was plotted with R; mode and mean value as well as the empirical standard deviation were calculated (Table S1).

Radiometric Saturation Binding Assay

The radioligand [³H]UR-MK292, a tritium labeled angiotensin-II derivative, was prepared according to a reported procedure^[86] with the following modification: As the supplier of the labeling reagent succinimidyl [³H]propionate (Novandi, Södertälje, Sweden) provides a precisely determined molar activity and due the fact that [³H]UR-MK292 bears exactly one tritiated propionyl residue originating from the labeling reagent, the molar activity of [³H]UR-MK292 was not determined by HPLC (calibration curve based on UR-MK292 and UV detection). Instead, it was defined to be equal to the molar activity of the labeling reagent, amounting to 3.44 TBq mmol⁻¹ (it is assumed that under the mild reaction conditions the carbon-tritium bonds remained intact). The molar activity of the labeling reagent succinimidyl [³H]propionate was determined by LC-MS analysis. Coinjection of “cold” succinimidyl propionate allowed the quantification of the incorporated tritium (Novandi, Södertälje, Sweden). [³H]UR-MK292 was obtained with a radiochemical yield of 39 %. The radiochemical purity amounted to 99 % (Figure S10). The synthesis of the “cold” equivalent (UR-MK292) was described previously.^[86]

For the saturation binding studies, the radioligand was stretched in a 1:1 ratio with cold ligand. Leibovitz's L-15 medium supplemented with bovine serum albumin (BSA) (0.1 %) and the protease inhibitor bacitracin (100 µg ml⁻¹) (Serva, Heidelberg, Germany) was used as binding buffer. For the experiments rMCs were cultured in T150 flasks (Corning, New York, USA) in passage number 78. On the day of experiment the cells were washed with DPBS, harvested with trypsin (PAN-Biotech, Aidenbach, Germany), and resuspended in binding buffer. 160 µL of the cell suspension were pipetted to each well of a 96-well plate (clear 96-well flexible PET microplate, round bottom, 1450 Beta, PerkinElmer), with the concentration adjusted to 250,000 cells/well. For total binding, binding buffer (20 µL) and the same volume of binding buffer containing the radioligand 10-fold concentrated, were added. The unspecific binding was determined by with the addition of binding buffer containing the radioligand (10-fold concentrated, 20 µL) and binding buffer containing EXP3174 in a 500-fold excess concentration as competitor (20 µL). The plates were incubated for 2 h at 22 ± 2 °C, gently shaken. Afterwards, the cells were quantitatively transferred to a filter mat (Whatman cytiva, GF/C™) that had been soaked with a 0.3 % polyethylenimine (PEI) solution beforehand, using a harvester for 96 well plates. The cells on the filter mat were washed twice with ice-cold PBS (200 µL). Filter pieces were punched out from the filter mat and were transferred into a 96-well plate (1450-401 96-well sample plate, PerkinElmer, Rodgau, Germany). 200 µL of scintillation cocktail (Rotiszint® Routine, NPE-free, Roth, Karlsruhe, Germany) were added and the plate was sealed with a transparent sealing tape (permanent seal for microplates, PerkinElmer). After incubation in the dark under

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shaking for at least 12 h, the radioactivity (dpm) was measured with a MicroBeta2 2450 microplate counter (PerkinElmer).

Quantification of the Selenium-Tagged Nanoparticles on the Cellular Matrix via ICP-MS

To quantify the number cell-associated nanoparticles when they were applied in the concentration of their K_i value, selenium-containing nanoparticles were produced. Their concentration was determined in independent triplicates by NTA measurements ($N = 3$; $n = 3$). Subsequently, the nanoparticle dispersion was diluted to the concentration of their K_i value (25 %-EXP-NPS_selenium-containing: 0.19 pM). RMCs, cultured in passage number 78, were harvested and their concentration was adjusted to $2.0 \cdot 10^6$ cells mL^{-1} in suspension with Leibovitz's medium. The target cells were incubated with the nanoparticles for 30 min at RT under moderate shaking. The experimental setting was chosen in terms of volume ratios of cell suspension to nanoparticles and incubation conditions as for the determination of the K_i of the nanoparticles (*please refer to Fura-2 AM-based Ca^{2+} mobilization assay*). After the incubation period, the supernatant was discarded by centrifugation. The selenium content in the cell pellets was measured using ICP-MS. In addition, a calibration curve of selenium-containing nanoparticles in the concentration range of 0.01 pM to 0.3 nM nanoparticle concentration was recorded. To compensate for the deviation in selenium content between different batches, which was calculated at 16.7 % (Figure S8), the calibration curve and the samples were prepared from the same batch of selenium-containing nanoparticles. The samples were prepared for ICP-MS measurement by adding $1 \mu\text{g L}^{-1}$ rhodium (1000 mg L^{-1} rhodium in 5 % HCl, Carl Roth GmbH, Karlsruhe, Germany) as internal standard and then measured according to DIN EN ISO 17294-2:2017-01 (LoQ = $1 \mu\text{g L}^{-1}$) on a NexION 2000 ICP-MS (Perkin Elmer, Waltham, Massachusetts, USA) operating with the software Syngistix 3.1 (2021). The unit was calibrated against a selenium standard containing 10 mg L^{-1} selenium (TraceCERT Periodic Table Mix 1).

Clustered Standard Error Analysis

Clustered standard error analyses were carried out for the regression models with R software.^[87] This accounts for the clustering that occurs in the data sets due to the multiple independent collection of regression lines and affects the standard errors, the t-statistics and the p-values of the averaged regression line.^[88] The corresponding regression tables report the estimators for intercept and gradient, the standard error as a measure of the precision of the estimates, the t-statistic and the p-value associated with the t-statistic.

Calculation Model

To experimentally determine the average number of simultaneously binding ligands per nanoparticle, the number of specific binding ligands (N_{bound}) was assumed to be a function of occupied receptors

(N_{oc}) relative to the number of nanoparticles (N_{NP}) in the system attached to the cell membrane (equation 10).

$$N_{bound} = \frac{N_{oc}}{N_{NP}}$$

eq. 10

If the number of nanoparticles present in the system corresponds to the concentration of their K_i value, it can be assumed that exactly half of the available receptors (N_{AT1R}) are occupied by ligands. N_{oc} can therefore be described as (equation 11):

$$N_{oc} = 0.5 \cdot N_{AT1R}$$

eq. 11

Substituting equation 11 into equation 10 yields equation 12, which can be solved by experimental data.

$$N_{bound} = \frac{0.5 \cdot N_{AT1R}}{N_{NP}}$$

eq. 12

The experimental data was collected with a polymer core-shell nanoparticle, whose surface was functionalized with a high affinity ligand. The ligand was losartan carboxylic acid (EXP3174), a full antagonist at the AT1R. Ligand-bearing chains and carboxy-terminated spacer chains in the shell of the model nanoparticle had the same chain length (kDa). The morphology of the nanoparticles was thus assumed to be “rigid tethered”. RMCs were chosen as a model cell line because of their stable AT1R expression.^[1] All assumptions refer to specific ligand-receptor interactions.

Fitting Models

The binding curves obtained from the Ca^{2+} mobilization assays with Lys-Ang-II, 25 %-EXP-NPs and 25 %-EXP-NPs_selenium-containing were fitted using a dose-response model (equation 13).

$$y = A1 + \frac{A2 - A1}{1 + 10^{(logIC50 - x) \cdot p}}$$

eq. 13

$A2$ indicates the maximum response asymptote and $A1$ the baseline, and p is the slope.

Data for saturation curves obtained from saturation binding studies with CY-5-labeled 25 %-EXP-NPs in flow cytometry experiments and from studies with [^3H]UR-MK292 were fitted using Michaelis-Menten binding model (equation 14), with B_{max} corresponding to the maximal binding capacity.

$$y = \frac{B_{max} \cdot x}{K_D + x}$$

eq. 14

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Density functions of nanoparticle size distributions were assessed by non-parametric Kernel density estimation.

The data was fitted using Origin 2022b software (OriginLab Corporation, Northampton, Ma, USA) and R software (R Core Team (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>).

Statistics

The statistical significance between means was evaluated with Student's two sample t-test and, in case of unequal variances, with Welch's two sample t-test using Origin 2022b software.

The effect of error propagation of the relative uncertainty of the results from different experiments (specified as SD and SEM) is difficult to estimate for the overall result. A particular focus was therefore on minimizing and characterizing the errors of the interim results.

Software

The software used for the different experiments is listed below.

TopSpin 4.1.4 software was used for NMR processing and analysis (Bruker Corporation, Billerica, MA, USA, URL: <https://www.bruker.com/en/products-and-solutions/mr/nmr-software/topspin.html>).

Origin 2022b software (OriginLab Corporation/Northampton/Ma/ USA. URL: <https://www.originlab.com/2022b>) was used for data plotting and analyzing.

Flowing software (v2.5.1, Cell Imaging and Cytometry Core, Turku Bioscience Centre, Turku, Finland, with the support of Biocenter Finland. URL <https://flowingsoftware.com/download/>) was used for FACS data analyses and plotting.

R software (R Core Team (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>) was used for data plotting and statistical analysis.

Gnuplot (Williams and Kelley. Gnuplot 5.4: an interactive plotting program. 2020. URL <http://gnuplot.info>. (Last accessed: 2022-11-16)) was used for data plotting.

Fiji software^[85] was used for TEM image processing.

Zen 3.5 blue edition software (Carl Zeiss Microscopy, Oberkochen, Germany) was used for confocal laser scanning microscopy image processing.

CorelDRAW 2023 Graphics Suite was used for graphical illustrations (Corel Corporation/Ottawa/Canada. URL <https://www.coreldraw.com/en/product/coreldraw/>).

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Chapter 3

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Supplementary Information

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K. S. (first author) designed and performed the research, analyzed the data und wrote the manuscript. All experiments and data that are not explicitly assigned to the contribution of other authors in the following are to be attributed to K.S.. S. B. provided scientific input to the project. O. Z. and F. J. B. contributed to the estimation of the receptor density in clathrin coated pits and generated Figure 8C. M. K. synthesized the radioligand, generated Figure S10A, wrote the Methods section describing the radioligand synthesis and characterization and provided scientific input to the quantification of receptor numbers on the target cell line. R. W. contributed to the acquisition of the TEM images and reviewed the final manuscript. A. G. supervised the project, provided scientific input during the complete process, supported the manuscript preparation and reviewed the final manuscript. The ICP-MS measurements were conducted by the INDIKATOR GmbH (Wilhelm-Muthmann-Str. 15, 42329 Wuppertal) on a contractual basis.

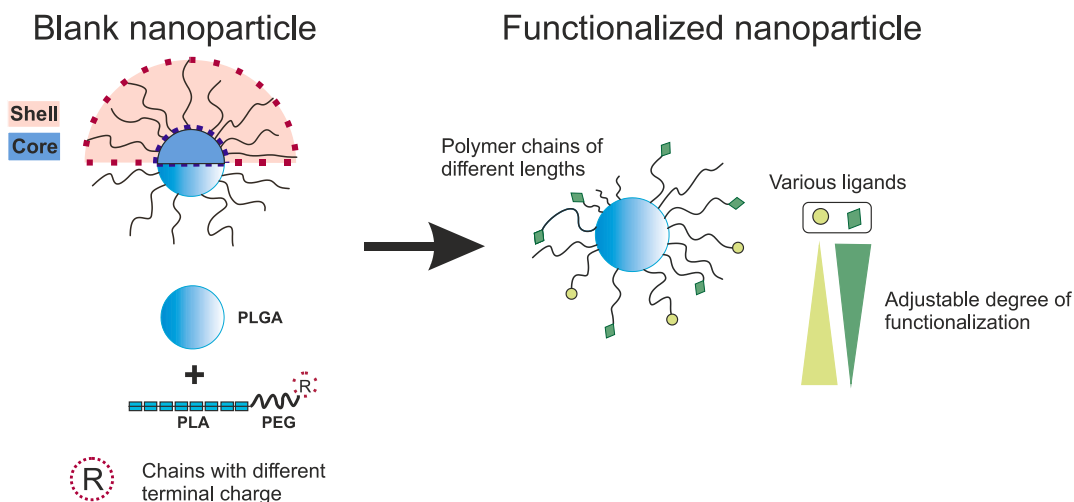


Figure S 1 Illustration of the nanoparticle design concept and its high degree of modularity in both polymer chain construction and functionalization. The polymer nanoparticles, well established in our group, ^[1–4] are composed as core-shell nanoparticles with biocompatible and biodegradable polymers selected for both, the shell, and the core. The use of PLGA in the core enables the incorporation of fluorescent dyes into the nanoparticle structure. PLA-PEG block copolymer chains can be synthesized with different molecular weights. They can be functionalized with a variety of small molecule or peptide ligands, whereby the degree of functionalization can be adapted to the target. Hetero-multivalent systems can be created by selecting combinations of ligands to address target cells. To achieve the highest possible target cell specificity and selectivity, the nanoparticle designs are frequently inspired by viral role models. The high degree of modularity in the nanoparticle design makes the implementation of complex targeting strategies feasible and the nanoparticles a promising platform technology, which is worth to be further investigated.

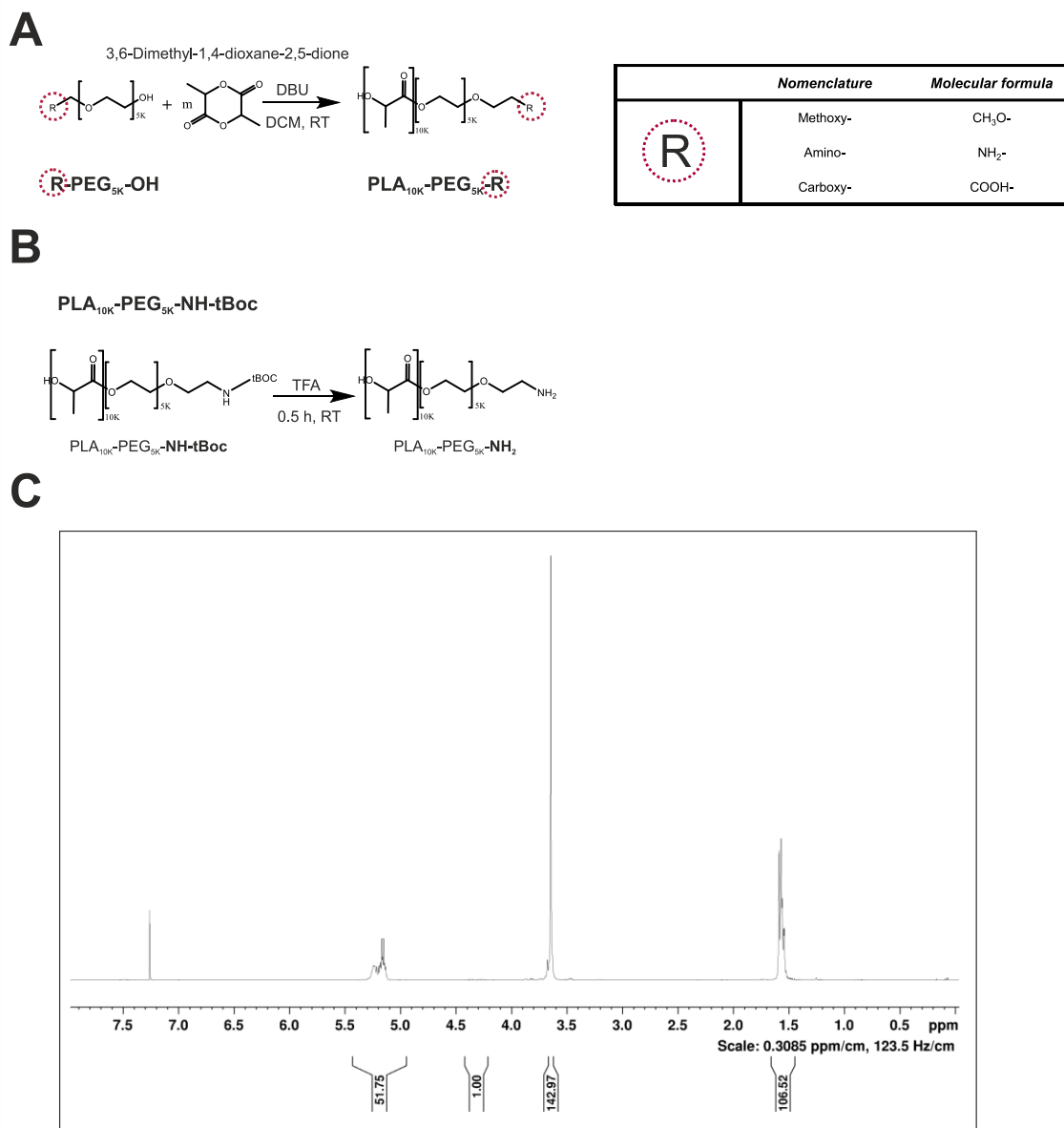


Figure S 2 Block copolymer synthesis, modification, and characterization. (A) Reaction equation for the synthesis of PEG-PLA block copolymers through ring opening polymerization of the cyclic lactone 3,6-dimethyl-1,4-dioxane-2,5-dione with PEG chains as macroinitiators for the reaction. The block copolymers can be synthesized with different terminal charges referred as head groups (R). (B) Reaction equation for the deprotection of *tert*-butoxycarbonyl (*tBoc*)-protected block copolymer. (C) Exemplary ^1H -NMR spectrum (400 MHz, CDCl_3) of $\text{NH}_2\text{-PEG}_{5K}\text{-PLA}_{10K}$ block copolymer: δ (ppm): 5.19 ppm (m, $-\text{C}(\text{CH}_3)\text{H-}$), 4.32 ppm (m, $-\text{OCH}_2\text{CH}_2\text{-O}(\text{CO})\text{-}$), 3.64 ppm (m, $\text{NH}_2\text{CH}_2\text{CH}_2\text{-}$), 1.57 ppm (m, $-\text{C}(\text{CH}_3)\text{H-}$).

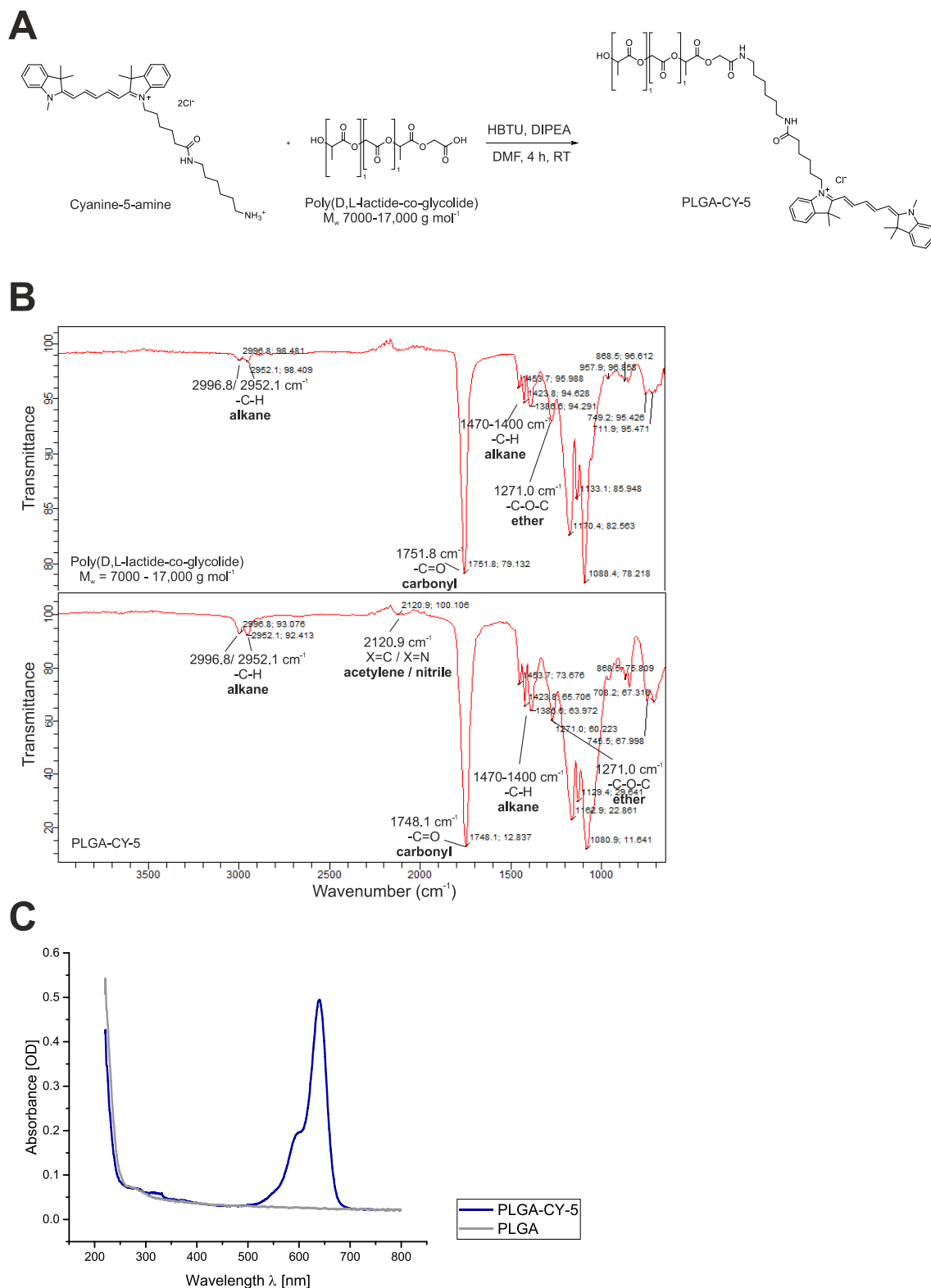


Figure S 3 Fluorescence labeling of PLGA with CY-5. (A) Reaction equation of fluorescence labeling of PLGA with CY-5. (B) FTIR spectra of PLGA (M_w 7000-17,000 g mol⁻¹) and PLGA-CY-5. A peak for acetylene/nitrile (2128.3 [cm⁻¹] ν (X=C/ X=N)) was found in the spectrum of PLGA-CY-5. (C) Absorbance spectra of PLGA and PLGA-CY-5 (4 mg mL⁻¹ in acetonitrile). The spectrum of PLGA-CY-5 exhibits a characteristic peak of CY-5 at a wavelength of λ 640 nm.

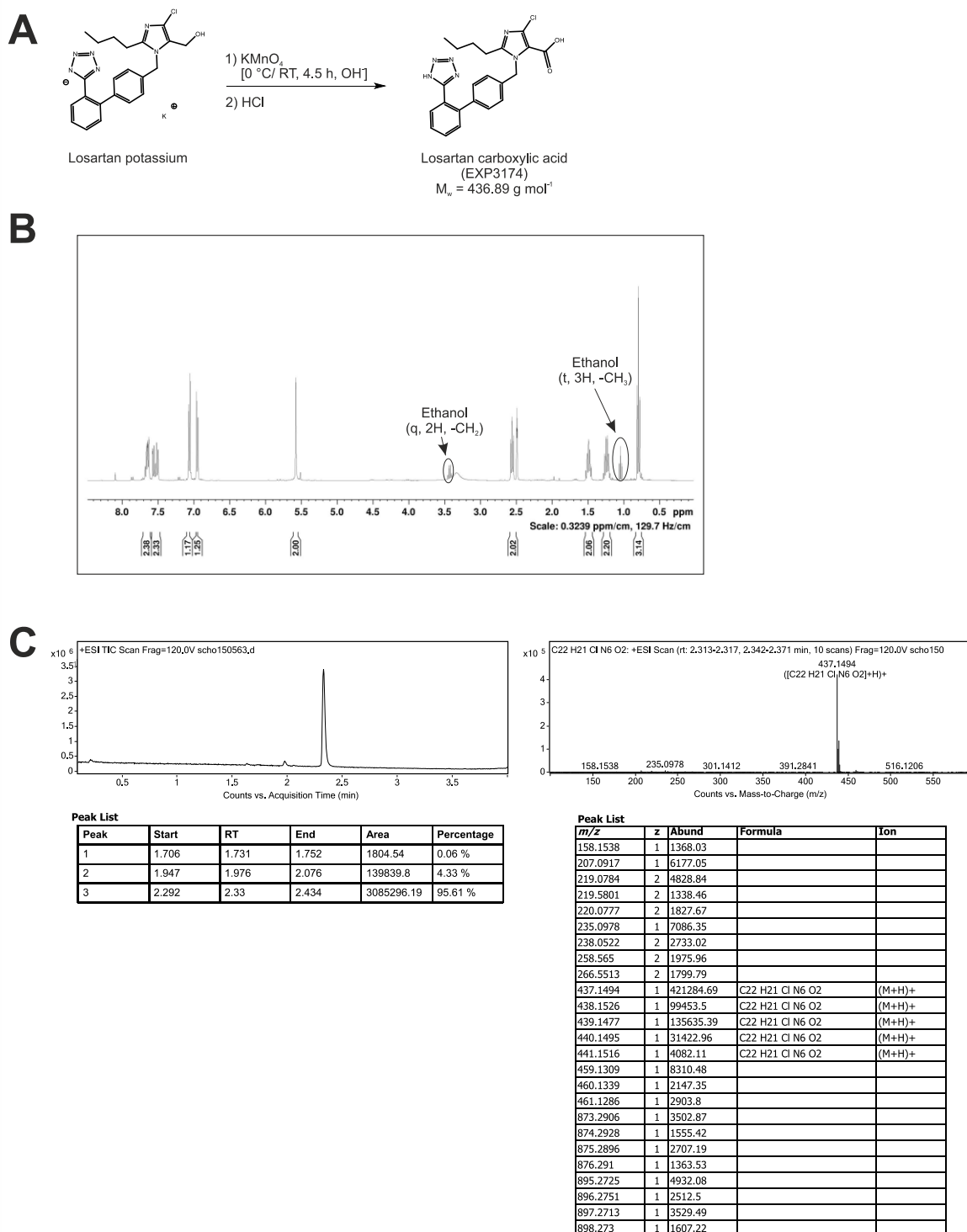


Figure S 4 Synthesis and confirmation of identity and purity of losartan carboxylic acid (EXP3174). (A) Reaction equation for the synthesis of EXP3174 obtained by oxidation of losartan with potassium permanganate. (B) ^1H -NMR spectrum (400 MHz, DMSO- d_6) of EXP3174: δ (ppm): 7.65 ppm (m, 2H, aromatic protons, ring B), 7.54 ppm (m, 2H, aromatic protons, ring B), 7.06 ppm (m, 1H, aromatic protons, ring A), 6.95 ppm (m, 1H, aromatic protons, ring A), 5.58 ppm (s, 2H, -NCH₂-), 2.56 ppm (t, 2H, -CH₂-), 1.48 ppm (m, 2H, -CH₂-), 0.80 ppm (t, 3H, -CH₃). Signals originating from residual ethanol were identified in the spectrum. δ (ppm): 3.43 ppm (q, 2H, -CH₂), 1.04 ppm (t, 3H, -CH₃). (C) LC-MS chromatogram showed purity of the product of > 95 %. A mass of 437.1494 (MH⁺) corresponding to EXP3174 was found.

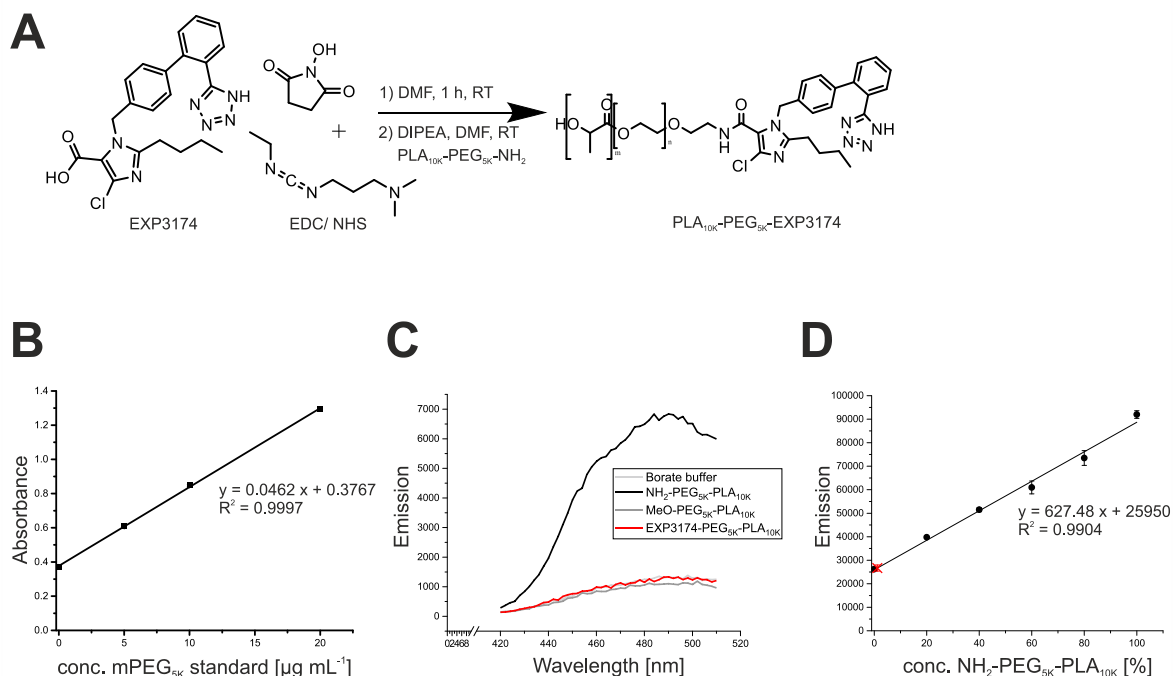


Figure S 5 Demonstration of the coupling efficiency of EXP3174 to the NH₂-PEG_{5K}-PLA_{10K} block copolymer. (A) Reaction equation for the coupling of EXP3174 to the polymer via EDC/NHS chemistry. (B) Preparation of polymer micelles of NH₂-PEG_{5K}-PLA_{10K}, MeO-PEG_{5K}-PLA_{10K} and EXP3174-PEG_{5K}-PLA_{10K} by nanoprecipitation for determining the degree of EXP3174-functionalization on the polymer. Calibration curve plotted against mPEG_{5K} standard to normalize the micelle concentrations according to their PEG content determined by Iodine assay. (C) Detection of free amino groups in the samples via Fluram assay. Emission spectra recorded after addition of borate buffer and fluorescamine to the samples (fixed excitation: λ 380/10 nm; emission start: λ 420/10 nm; emission stop: λ 510 nm; step: 2 nm; recorded on a Synergy Neo 2 Multi-Mode Microplate Reader). In addition, the sample background was captured by recording an emission spectrum of borate buffer after addition of fluorescamine under the same measurement conditions. The measured emission of the EXP3174-functionalized polymer was in the order of magnitude of the background and the amino group free MeO-PEG_{5K}-PLA_{10K} micelles. (D) Calibration line plotted for the concentration of free amino groups by measuring the fluorescence of methoxy- and amino-terminated polymer micelles at specific concentration ratios (amino- to methoxy-terminated, 100:0 / 80:20 / 60:40 / 40:60 / 20:80 / 0:100) after addition of fluorescamine. The calculated content of free amino groups of the EXP3174-functionalized polymer was less than 2 % (degree of EXP3174-functionalization > 98 %). The value is marked as a red cross on the calibration line. Results (B-D) represented as mean $\pm$ SD ($n = 3$).

Characterization of the PEG Conformation and Validation of the Nanoparticle Morphology

To validate the rigid, tethered and spherically enclosed morphology of both model nanoparticle types, the conformation of the PEG chains in the nanoparticle shell was determined. Therefore, the Flory radius (R_F), which corresponds to the radius of the semispherical volume of a PEG polymer, was calculated. R_F was compared to the distance between two grafting sites, D , as it has been already described by our group and others.^[9,10] R_F is defined in equation 1, where $\alpha = 0.35$ nm corresponds to the length of a PEG monomer and $[N]$ is calculated as the number of monomers in one PEG molecule by dividing the molecular weight of the PEG chain (5000 g mol^{-1}) by the monomer's molecular weight of 44 g mol^{-1} .

$$R_F = \alpha \cdot [N]^{\frac{3}{5}}$$

eq. 1

R_F was calculated to be 6.0 nm for the PLA_{10K}-PEG_{5K} block copolymer chains used for the nanoparticle design. D was calculated for both nanoparticle types as shown in equation 2, where S corresponds to the surface area that each PEG chain occupies on the nanoparticle surface^[11] and is separately defined in equation 3.

$$D = 2 \cdot \sqrt{\frac{S}{\pi}}$$

eq. 2

$$S = \frac{6 \cdot M_{PEG}}{dh \cdot N_A \cdot f \cdot \rho_{NP}}$$

eq. 3

According to equation 3, S is defined by the molecular weight of a PEG chain (M_{PEG}), which is related to the (hydrodynamic) diameter of the nanoparticles (dh), the mass fraction of PEG in the blends of the PLA_{10K}-PEG_{5K} block copolymer (f), and the density of the nanoparticles (ρ_{NP}). A density of 1.25 g cm^{-3} was assumed as a rough estimate for the density of the nanoparticles. The value corresponds to the density of nanoparticles composed of PLA-g-PEG blends.^[12]

We considered this justified under the assumption that S is a circular area, and that the polymer distribution is homogeneous on the nanoparticle surface. D was calculated for both nanoparticle types (25 %-EXP-NPs and 25 %-EXP-NPs_selenium-containing) to be 1.3 nm. Since R_F was greater than D , it could be assumed that the polymer chains adopted a brush conformation. The model assumption of rigid, tethered and spherically enclosed nanoparticles was validated by this calculation.

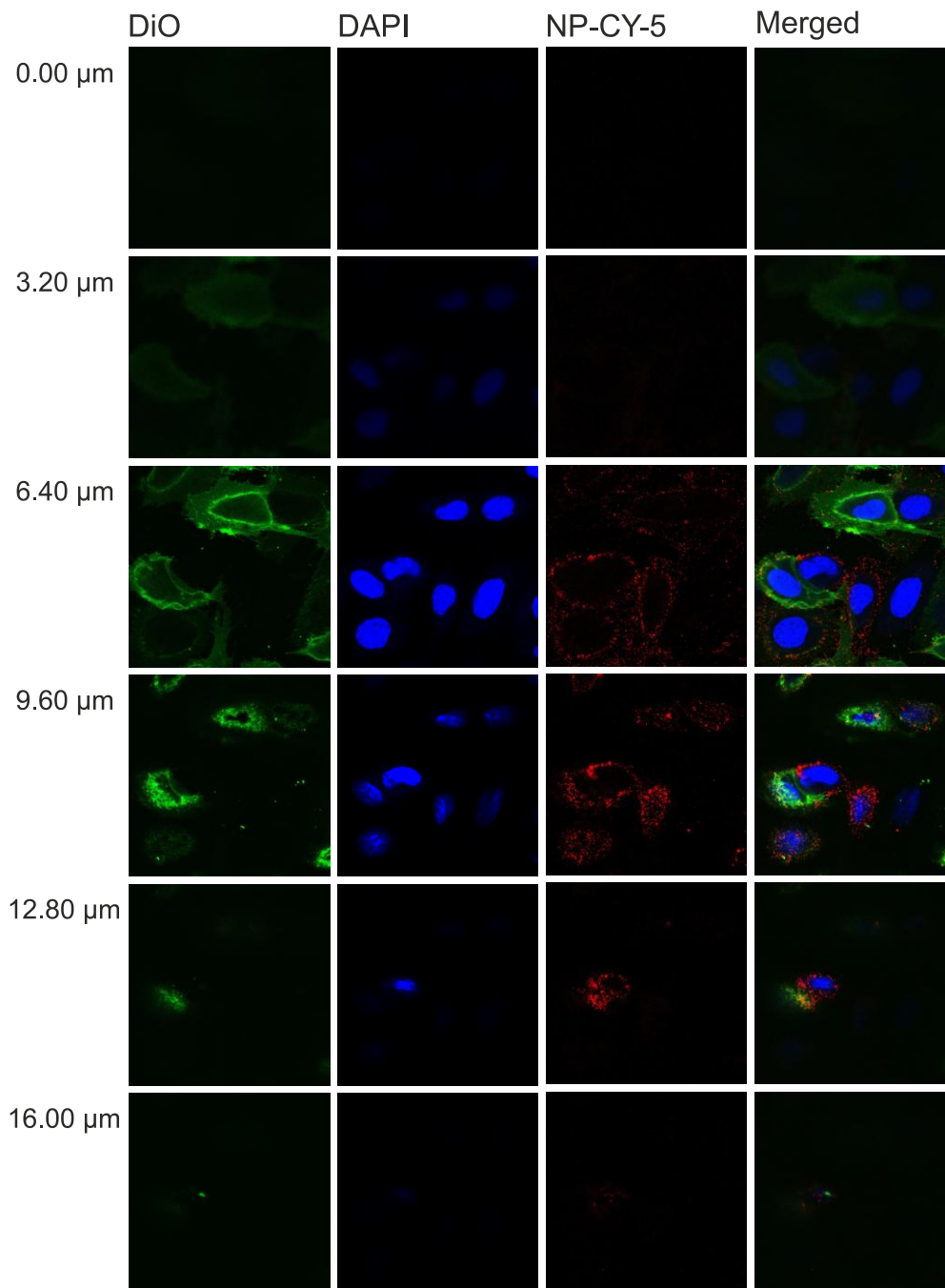


Figure S 6 Confocal laser scanning microscopy Z-stack images of rMCs incubated with CY-5-labeled nanoparticles (3 nM) at RT to prove the particle localization on the cell surface. 25 %-EXP-NPs were obtained by nanoprecipitation and fluorescently labeled using CY-5 tagged PLGA. The nanoparticle dispersion was adjusted to 3 nM in Leibovitz's L-15 medium and rMCs were incubated with 200 μ L of the dispersion for 30 min at RT (22 $^{\circ}$ C). Cell fixation was performed with paraformaldehyde. Afterwards, cell nuclei were stained with DAPI and cell membranes with DiO. Z-stack images (6 stacks of 3.20 μ m) were recorded on a Zeiss LSM 710 (Carl Zeiss Microscopy GmbH, Jena, Germany). Images were processed and analyzed using ZEN 3.5 blue edition software. The 25 %-EXP3174 functionalized nanoparticles were mainly localized on the cell membrane, as evident from the merged channel.

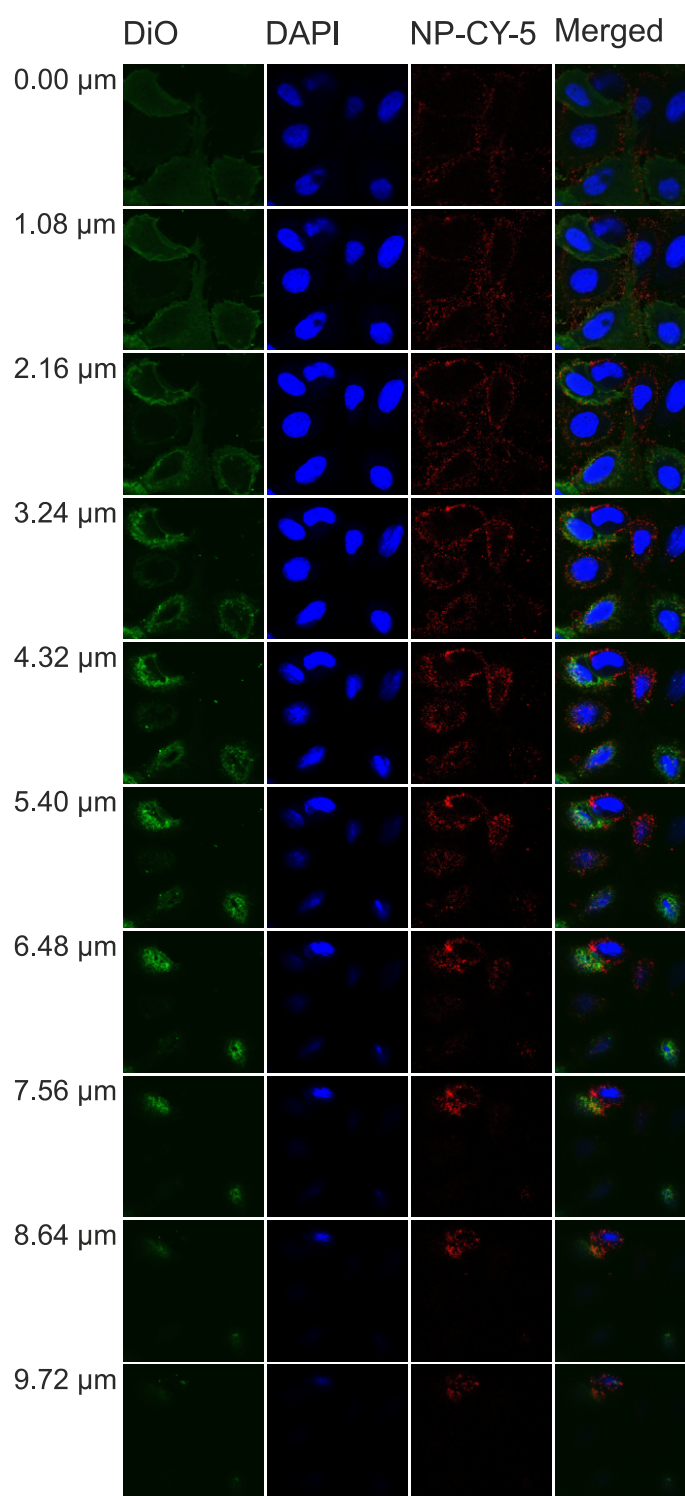


Figure S 7 Confocal laser scanning microscopy Z-stack images of rMCs incubated with CY-5-labeled nanoparticles (3 nM) at RT to prove the particle localization on the cell surface. Further Z-stack images (10 stacks of 1.08 μm) were recorded on the Zeiss LSM 710 (Carl Zeiss Microscopy GmbH, Jena, Germany) for fine resolution. The samples were prepared according to the procedure described above (Figure S6). Images were processed and analyzed using ZEN 3.5 blue edition software. The 25 %-EXP3174 functionalized nanoparticles were mainly localized on the cell membrane, as evident from the merged channel.

ICP-OES Measurements

ICP-OES measurements were carried out on a Spectroblue ICP-OES (SPECTRO Analytical Instruments GmbH, Kleve, Germany) operating with the software Smart Analyzer Vision. The unit was calibrated against a selenium standard containing 1.000 g L^{-1} selenium (Bernd Kraft GmbH, Duisburg, Germany) in a concentration range from 100 to 16000 ppb. Selenium-containing nanoparticles were produced according to the procedure described in the experimental section. The samples were prepared for measurement by acidic digestion.^[5,6] To remove the organic matrix, they were heated in 350 μL of concentrated nitric acid (65 %) (Fisher Scientific, Loughborough, UK) for two hours at 75 °C in a water bath. Subsequently, the tubes were cooled down and the samples were diluted in Milli-Q water to a final acidic concentration of 1 M nitric acid. A series of calibrations lines in the concentration range of 0.1 pM to 0.3 nM nanoparticle concentration was recorded to demonstrate successful implementation of selenium in the polymeric matrix and to calculate the batch-to-batch deviation in their selenium content. The recorded calibration lines were merged under consideration of the clustering in the data structure.

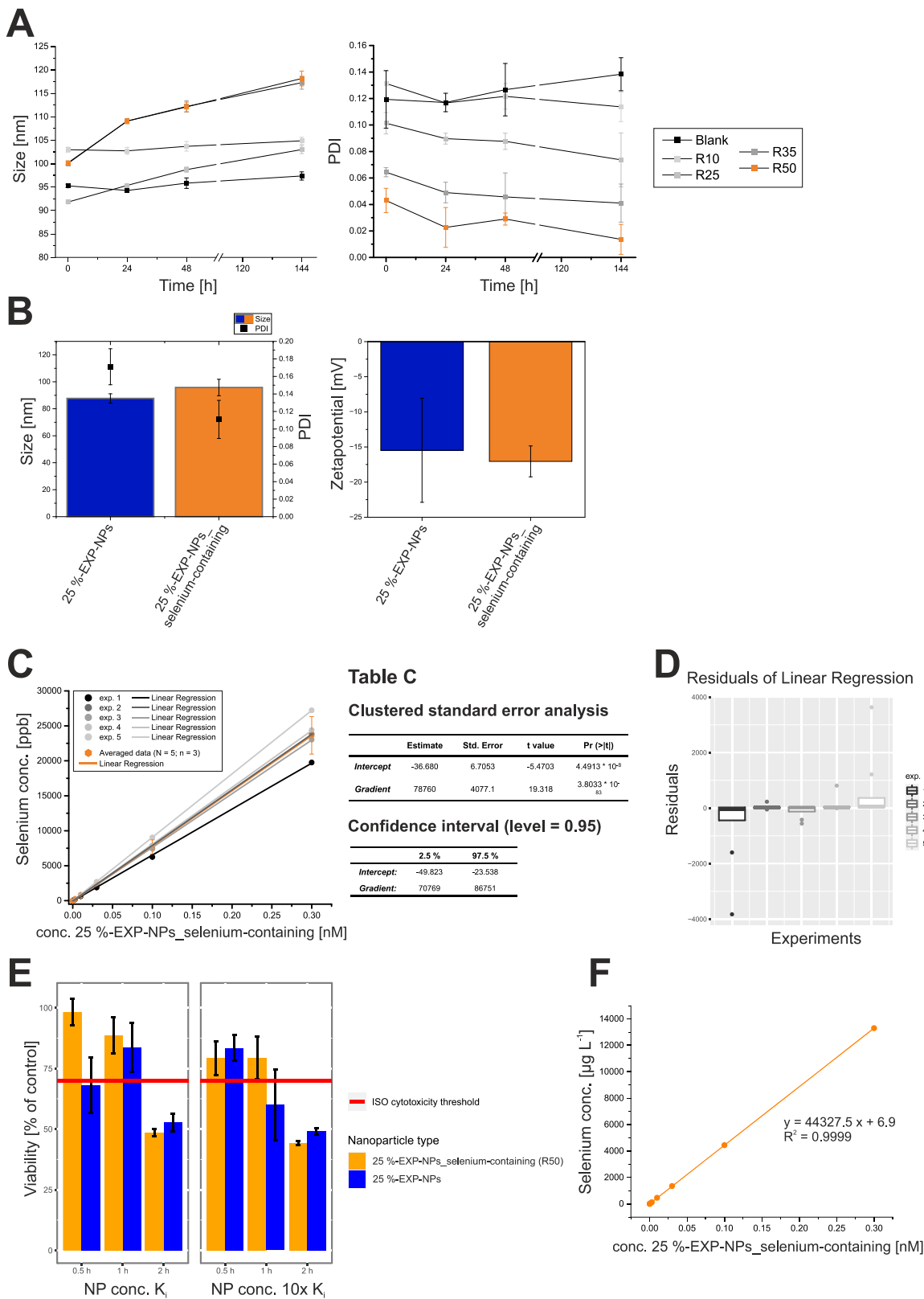


Figure S 8 Implementation of selenium as an ICP-OES and ICP-MS tag into the polymer matrix of the model nanoparticles. (A) For the formulation trials of selenium-containing nanoparticles, stability studies on size and PDI, measured by DLS, were conducted on the day of nanoparticle preparation, after 24 h, after 48 h and after 144 h. The data were determined on non-functionalized blank nanoparticles (core: PLGA; shell: PLA_{10K}-PEG_{5K}-COOH). We consider this justified as the ligand is not expected to influence the amount of

stabilizable selenium. Nanoparticles produced without addition of selenium were used as control (Blank). In the formulation trials, the ratio of selenium to PLGA was related as an excess to the ratio of 10.64 mg g⁻¹ selenium to polymer published by Luo et al.^[7] as successfully stabilized. (E.g., R50 describes a ratio of 532 mg g⁻¹ selenium related to the amount of PLGA used to produce the particles.) All nanoparticle batches showed increasing size over the course of the study (data shown as mean $\pm$ SD, n = 3). As the amount of stabilized selenium increased, the PDI of the batches decreased (data shown as n = 3, mean $\pm$ SD). The target formulation, chosen for the further experiments, was marked in orange (R50). (B) Nanoparticle data on hydrodynamic diameters, polydispersity index (PDI) and zeta potential obtained from dynamic light scattering (DLS) measurements. Results represented as mean $\pm$ SD (N = 5; n = 3). The data sets were tested for normal distribution using the Shapiro-Wilk normality test and for homogeneity of variance using the F-test. Depending on homogeneity of variance the data sets were compared using either Student's two-sample t-test or Welch's two-sample t-test. The two nanoparticle types differed by less than 10 nm in their size (25 %-EXP-NPs: 87.71 $\pm$ 3.42 nm; 25 %-EXP-NPS_selenium-containing: 95.82 $\pm$ 6.11 nm; Welch's two-sample t-test, Pr(>t) = 1.872 $\cdot$ 10⁻⁴) and had significantly different PDIs (25 %-EXP-NPs: 0.171 $\pm$ 0.021; 25 %-EXP-NPS_selenium-containing: 0.111 $\pm$ 0.022; Student's two-sample t-test, Pr(>t) = 1.813 $\cdot$ 10⁻⁸). We hypothesized that the amorphous selenium served as crystallization nucleus for the polymer matrix. It could thus contribute to a narrower size distribution, but to a larger particle size in absolute terms. Due to the carboxy-terminated spacer chains, both nanoparticle types showed negative and comparable zeta potentials (25 %-EXP-NPs: -15.48 $\pm$ 7.40 mV; 25 %-EXP-NPS_selenium-containing: -17.08 $\pm$ 2.12 mV; Welch's two-sample t-test, Pr(>t) = 0.432). (C) A series of independent calibration lines in the concentration range of 0.1 pM to 0.3 nM nanoparticle concentration of the target formulation (R50) was recorded on a Spectroblue ICP-OES (SPECTRO Analytical Instruments GmbH, Kleve, Germany). The values were pooled to a calibration curve (orange). Clustering in the data due to multiple independent measurements was accounted for by processing the data sets with a clustered standard error analysis. The intercept and gradient, together with the corresponding standard errors, were summarized in Table C. The confidence interval of the calibration line was calculated at the level of 0.95 (Table C). The negative intercept indicated a limitation of the quantifiability of the selenium content by ICP-OES in the lower concentration range. The deviation of the individual batches in their selenium content was calculated at 16.7 %. (D) Plotted residuals of the individual data points to the pooled calculated calibration line. (E) The cytotoxicity of 25 %-EXP-NPs and 25 %-EXP-NPs_selenium-containing (R50) on target cells was determined using MTT-assay.^[3,8] In brief, rMCs were seeded at a density of 15,000 cells/well in a 96-well plate and cultured for 24 h (37 °C, 5 % CO₂). Both types of nanoparticles were manufactured and diluted with Leibovitz's L-15 medium. The target cells were incubated for 0.5 h, 1 h and 2 h with nanoparticles adjusted to the concentration of their K_i value and their 10-fold K_i value (10x K_i). RMCs incubated with Leibovitz's L-15 medium served as control. After the incubation period, the samples were discarded and 200 μ L of a 1 mg mL⁻¹ 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dilution in RPMI medium were added and incubated for 3 h. Afterwards, the MTT dilution was aspired and 200 μ L isopropanol was added. The wells were incubated under gentle shaking and light exclusion for 30 min at RT. Subsequently, the absorbance was measured at λ 570 nm and λ 690 nm on a FLUOstar Omega plate-reader (BMG Labtech, Ortenberg, Germany). The viability was assessed using the difference in absorbance at both wavelengths to the control. Results represented as mean $\pm$ SD (N = 3; n = 3). In the test-relevant concentrations (K_i / 10x K_i) and test-relevant time frame (0.5 h), the viability in relation to the control was above the threshold of 70 % specified by the ISO standard (ISO 10993-5:2009 (*Biological evaluation of medical devices, part 5: Tests for in vitro cytotoxicity*)) for both nanoparticle types. (F) A calibration line of 25 %-EXP-NPs_selenium-containing was recorded in the concentration range of 0.1 pM to 0.3 nM nanoparticle concentration using ICP-MS analytics.

		Size (dh/d) [nm]			Surface (O _{NP}) [nm ²]			Ligand valence (N _L)			Ligands/100 nm ²
		mean	SD	mode	mean	SD	mode	mean	SD	mode	mean
25 %-EXP-NPs	NTA	81.7	17.5	81.5	21947.9	10071.5	20867.2	4112	3199	3555	19
	DLS	81.1	30.0	78.8	23499.1	18133.0	19517.4	5096	6253	3216	22
	TEM	40.7	13.0	37.1	5747.3	4099.1	4334.4	598	759	337	10
25 %-EXP-NPs_ selenium-containing	NTA	95.1	20.6	82.5	29724.3	20628.2	21382.5	6721	15061	3687	23
	DLS	107.8	39.1	105.7	41320.8	31160.0	35099.4	11804	14093	7755	29
	TEM	47.4	15.0	35.7	7776.0	5351.5	4002.4	932	1133	299	12

Table S 1 Representation of the size (dh/d) [nm], the calculated surface area and the mean ligand valence of the two particle types, 25 %-EXP-NPs and 25 %-EXP-NPs_selenium-containing, as a function of the measurement method used. Data are represented as mean $\pm$ SD and mode. NTA (NanoSight NS300, Malvern, Worcestershire, UK) and DLS (Malvern Zetasizer Nano ZS, Malvern Instruments, Herrenberg, Germany) raw data were obtained in a triplicate measurement (n = 3). The DLS data was analyzed as intensity percent. The size of 500 nanoparticles was measured on six independent sections of each TEM grid.

		NTA concentration measurement	
		Fraction of binding ligands [%]	Binding ligands/100 nm ²
25 %-EXP-NPs	NTA	7.5	1.3
	DLS	8.7	1.4
	TEM	65.1	5.3
25 %-EXP-NPs_ selenium-containing	NTA	4.7	0.9
	DLS	3.7	0.8
	TEM	41.3	3.9

Table S 2 Maximum fraction of binding ligands and number of binding ligands per 100 nm² nanoparticle surface area (data shown as geometric mean). DLS data was analyzed as intensity percent. For very small nanoparticles in the size distributions, the calculated average binding ligand number exceeded their ligand valence. From a mathematical point of view, this led to values > 1, which were not excluded from the calculation of the geometric mean.

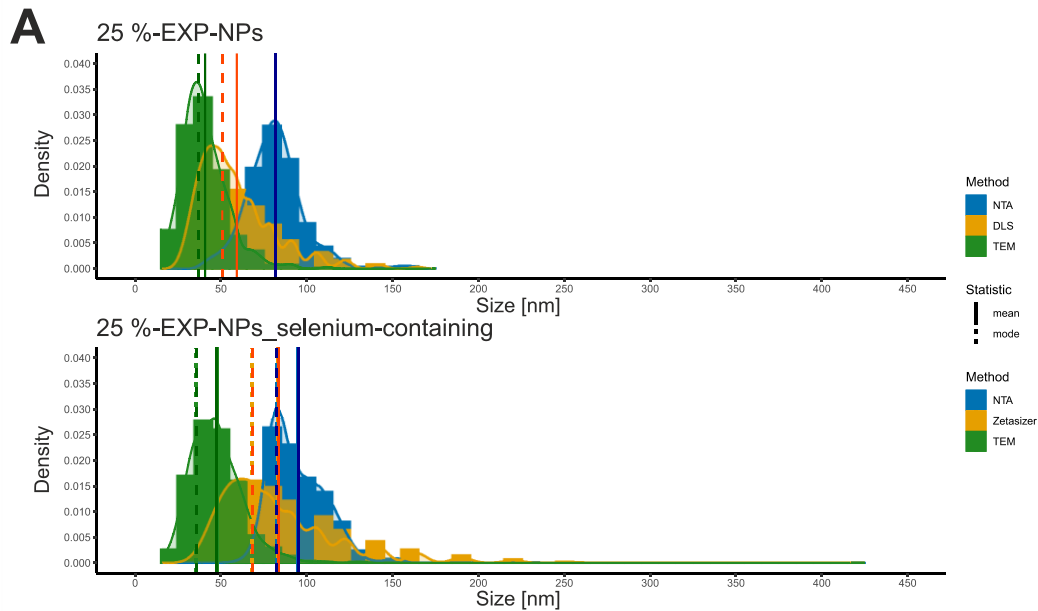


Table A1

		Size (dh/d) [nm]			Surface (O _{NP}) [nm ²]			Ligand valence (N _L)			Ligands/100 nm ²
		mean	SD	mode	mean	SD	mode	mean	SD	mode	mean
25 %-EXP-NPs	NTA	81.7	17.5	81.5	21947.9	10071.5	20867.2	4112	3199	3555	19
	DLS	59.3	22.5	50.8	12611.3	11027.2	8091.4	2064.7	3188.2	858.3	16
	TEM	40.7	13.0	37.1	5747.3	4099.1	4334.4	598	759	337	10
25 %-EXP-NPs_selenium-containing	NTA	95.1	20.6	82.5	29724.3	20628.2	21382.5	6721	15061	3687	23
	DLS	83.2	34.5	68.1	25495.9	24468.8	14552.4	6143.6	10402.4	2070.3	24
	TEM	47.4	15.0	35.7	7776.0	5351.5	4002.4	932	1133	299	12

Table A2

		NTA concentration measurement	
		Fraction of binding ligands [%]	Binding ligands/100 nm ²
25 %-EXP-NPs	NTA	7.5	1.3
	DLS	22.1	2.6
	TEM	65.1	5.3
25 %-EXP-NPs_selenium-containing	NTA	4.7	0.9
	DLS	8.2	1.3
	TEM	41.3	3.9

Figure S 9 Characterization of size and ligand distribution of the model nanoparticles (DLS data shown and analyzed as volume percent). (A) Size distribution curves of 25 %-EXP-NPs and 25 %-EXP-NPs_selenium-containing plotted as a function of the measurement method. Size values obtained by NTA, DLS, and TEM measurements were compared. Table A1 represents the size (*dh/d*), the calculated surface area and the mean ligand valence of the two particle types, 25 %-EXP-NPs and 25 %-EXP-NPs_selenium-containing, as a function of the measurement method used. Table A2 represents the maximum fraction of binding ligands and number of binding ligands per 100 nm² nanoparticle surface area (data shown as geometric mean).

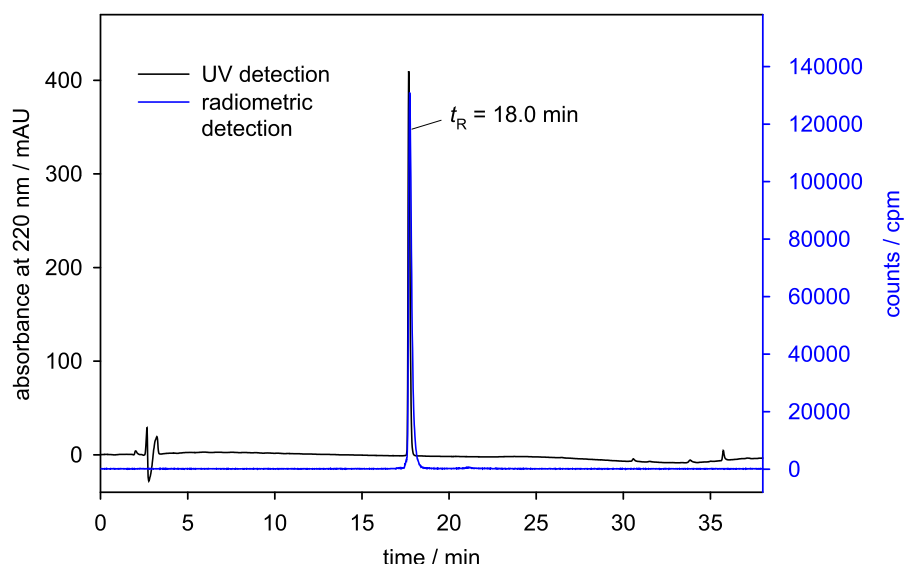
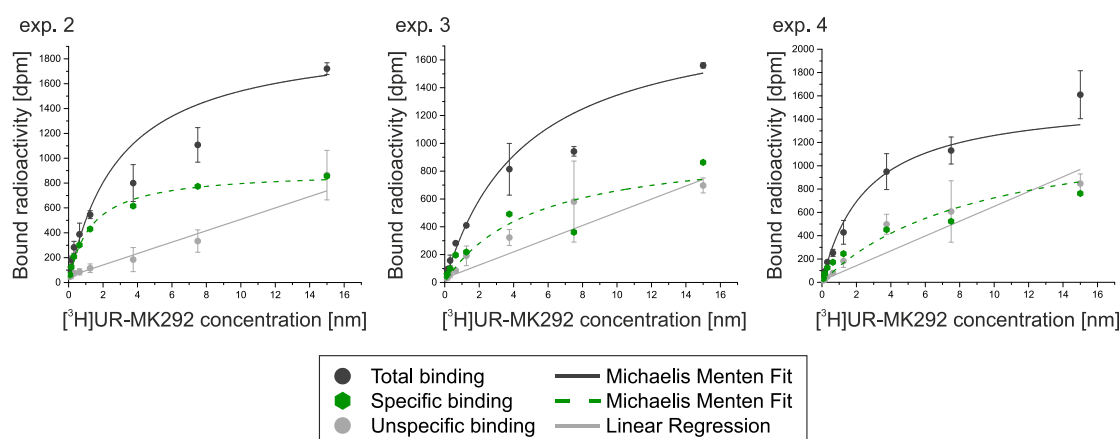
A**B**

Figure S 10 Determination of the radiochemical purity of $[^3\text{H}]\text{UR-MK292}$ and radiochemical binding studies with the radioligand on rMCs. (A) RP-HPLC analysis of $[^3\text{H}]\text{UR-MK292}$ spiked with “cold” UR-MK292, on a system from Waters (Eschborn, Germany) consisting of two pumps 510, a pump control module, a 486 UV/VIS detector, a manual injector, and a Flow-one beta series A-500 radiodetector (Packard, Meriden, USA). Column: Luna C18(2) 150×4.6 mm, $3 \mu\text{m}$ (Phenomenex, Aschaffenburg, Germany). Mobile phase: linear gradient, 0–20 min: acetonitrile + 0.04 % TFA/water + 0.05 % TFA 10:90–38:42, 20–32 min: 38:42–95:5, 32–38 min: 95:5 (isocratic). Flow: 0.8 mL/min. Injection: 100 μL of a solution containing $[^3\text{H}]\text{UR-MK292}$ (0.2 μM) and UR-MK292 (20 μM). The radiochemical purity of $[^3\text{H}]\text{UR-MK292}$ was 99 %. (B) Radiochemical saturation binding studies with $[^3\text{H}]\text{UR-MK292}$ performed to determine the average number of AT1R per cell on rMCs. Data obtained from independent experiments (exp. 2 - 4) (mean $\pm$ SD; $n = 3$).

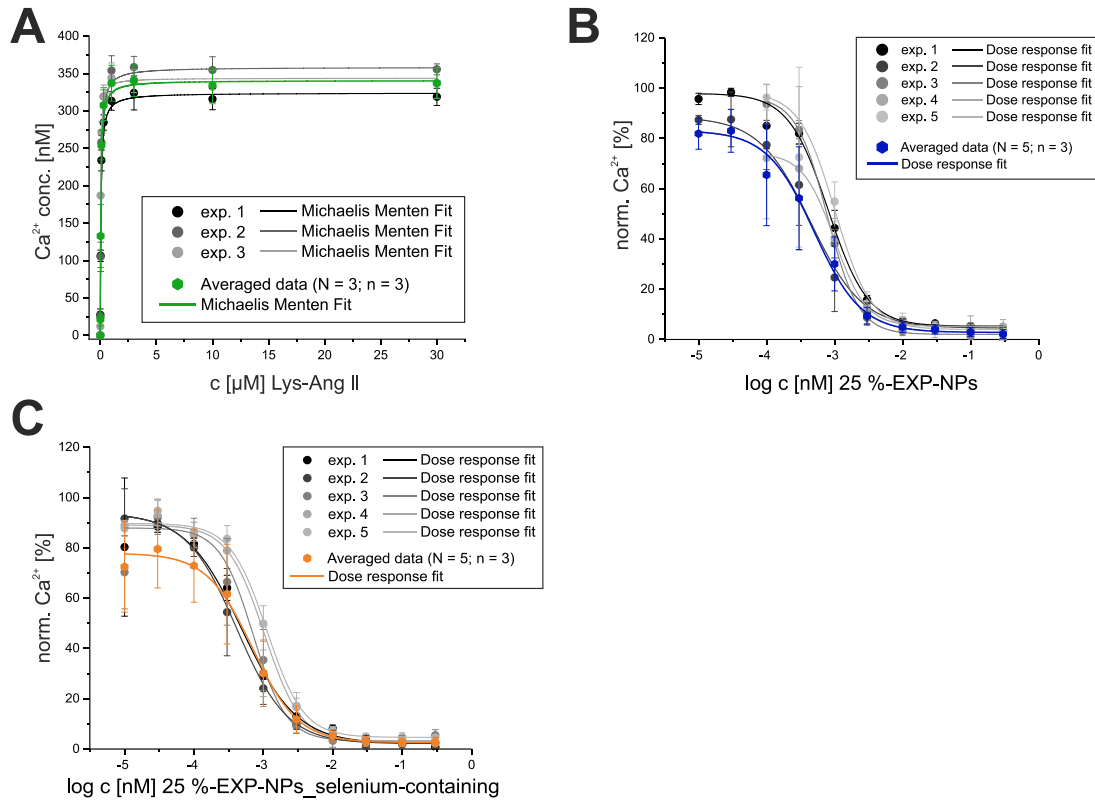


Figure S 11 Saturation and binding curves derived from Fura-2 AM based Ca^{2+} mobilization assay. (A) Saturation curves derived from data acquired for Lys-Ang-II on Fura-2 AM loaded rMCs. The data sets were obtained from three independent experiments ($N = 3$; $n = 3$), with the averaged binding curve shown in green and error bars reflecting standard deviation. (B) Serial dilutions of 25 %-EXP-NPs were prepared and measured on Fura-2 AM loaded rMCs. The binding curves were derived from five independent experiments ($N = 5$; $n = 3$), with the averaged binding curve shown in blue and error bars reflecting standard deviation. (C) 25 %-EXP-NPs_selenium-containing were manufactured, and binding curves were obtained from five independent experiments ($N = 5$; $n = 3$), recorded on Fura-2 AM loaded rMCs. The averaged binding curve is shown in orange, the error bars represent standard deviation.

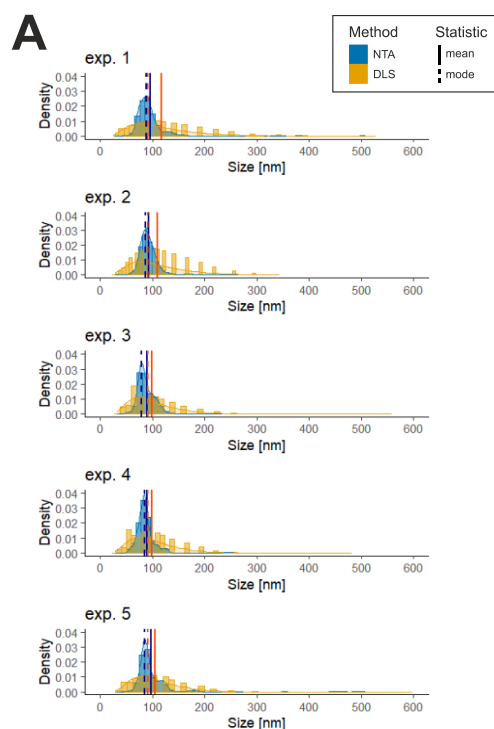


Table A

	25 %-EXP-NPs_selenium-containing					
	NTA dh (nm)			DLS dh (nm)		
	Mean	SD	Mode	Mean	SD	Mode
exp.1	98.7	31.4	94.5	104.7	34.6	91.3
exp. 2/3	85.2	25.8	77.5	120.4	44.8	105.7
exp. 4	91.8	26.8	92.5	108.5	39.1	105.7
exp. 5	93.1	26.4	87.5	108.5	37.0	105.7
Inter-batch SD:	5.5		7.6	6.8		7.2

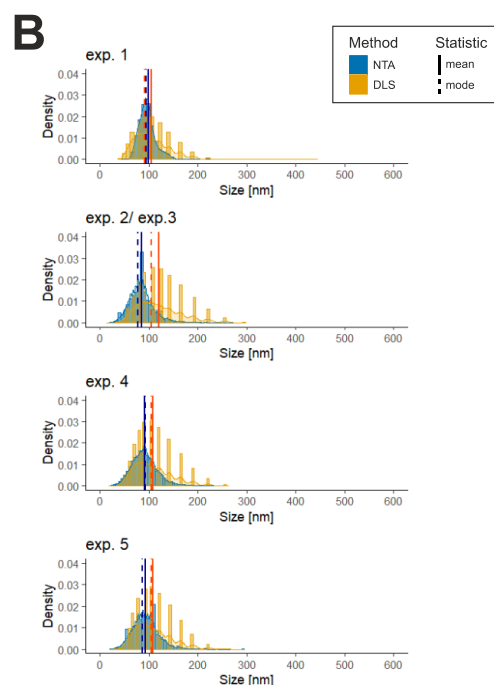


Table B

	25 %-EXP-NPs					
	NTA dh (nm)			DLS dh (nm)		
	Mean	SD	Mode	Mean	SD	Mode
exp.1	96.0	39.8	88.5	117.1	61.9	91.3
exp. 2	92.3	17.9	86.5	110.2	53.5	91.3
exp. 3	89.3	30.3	79.5	99.4	43.0	91.3
exp. 4	89.3	33.9	84.5	99.5	41.8	91.3
exp. 5	98.0	41.0	85.5	105.4	44.2	91.3
Inter-batch SD:	3.9		3.4	7.5		0

Figure S 12 Size distributions curves of the nanoparticle batches used to determine the K_i values, obtained from NTA (NanoSight NS300, Malvern, Worcestershire, UK) and DLS measurements (Malvern Zetasizer Nano ZS, Malvern Instruments, Herrenberg, Germany). Data were obtained from triplicate measurements ($n = 3$). (A) 25 %-EXP-NPs and (B) 25 %-EXP-NPs_selenium-containing were produced according to the procedure already described. The size distribution of the nanoparticles was measured independently for each batch by NTA and DLS. The obtained data were shown as mean $\pm$ SD and mode in the Tables A and B.

Considerations on Clathrin Coated Pit Geometry

The geometry of CCPs was simplified to a spherical dome, whose surface area (O_{CCP}) can be calculated using equation 4. Here r refers to the radius of the sphere, a is the radius of the base circle of the spherical segment and h is the height of the spherical dome. The relationship between these variables is shown in equation 5.

$$O_{CCP} = \pi(2rh + a^2)$$

eq. 4

$$r^2 = (r - h)^2 + a^2 \leftrightarrow r = \frac{h^2 + a^2}{2h}$$

eq. 5

As the CCPs differ in their diameter, d , and depth, h , depending on their stage of development,^[13,14] the parameter ε was introduced to accommodate this (equation 6).

$$\varepsilon = \frac{d}{h} = \frac{2a}{h}$$

eq. 6

The geometry of the AT1R was described by the simplified model of a cylinder with the radius r_R . In a previous study, we have calculated the diameter of a single AT1R to be 4.6 nm, and r_R therefore to be 2.3 nm.^[10] The membrane area occupied by a single receptor can be determined by equation 7.

$$A_{AT1R} = \pi \cdot r_R^2$$

eq. 7

However, this would require that there is no spacing between the individual receptors on the cell membrane. Aldossary et al.^[15] have recently published a study in which they analyzed the distribution and clustering of AT1R on rat pheochromocytoma cells (PC12 cells). This study provided at least a good approximation of the rat mesangial cells we used due to the origin from the same species. They demonstrated a cluster density of $2.2 \cdot 10^4$ localizations per μm^2 in AT1R clusters on the cell surface (data represents the mode). Based on their published data, we calculated the minimum area occupied per receptor to be 3.6 nm. The membrane area occupied by a single receptor was therefore described as a function of the radius of the receptor (2.3 nm) and the buffer area, r_B , between the receptors (1.3 nm), as shown in equation 8.

$$A_{AT1R} = \pi \cdot (r_R + r_B)^2$$

eq. 8

Based on the assumptions made, the number of AT1R per clathrin coated pit could be estimated by dividing the surface area of the CCP by the membrane area reserved for a single AT1R, as shown in

equation 9. For a more accurate estimation, a correction factor for the closest packing of circles (0.907) had to be included in the equation.

$$N_{AT1R/CCP} = \frac{O_{CCP} \cdot 0.907}{A_{AT1R}} = \frac{(\pi(2rh + a^2)) \cdot 0.907}{\pi \cdot (r_R + r_B)^2}$$

eq. 9

Substituting equations 4-6 into equation 9 yields the following function (equation10) for the number of AT1R depending on ε , a , and h of the respective CCP.

$$N_{AT1R/CCP}(\varepsilon, a, h) = \frac{\left(\pi \left(\frac{\varepsilon^2 h^2}{4} + h^2 + a^2 \right) \right) \cdot 0.907}{\pi \cdot r_R^2}$$

eq. 10

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Chapter 4

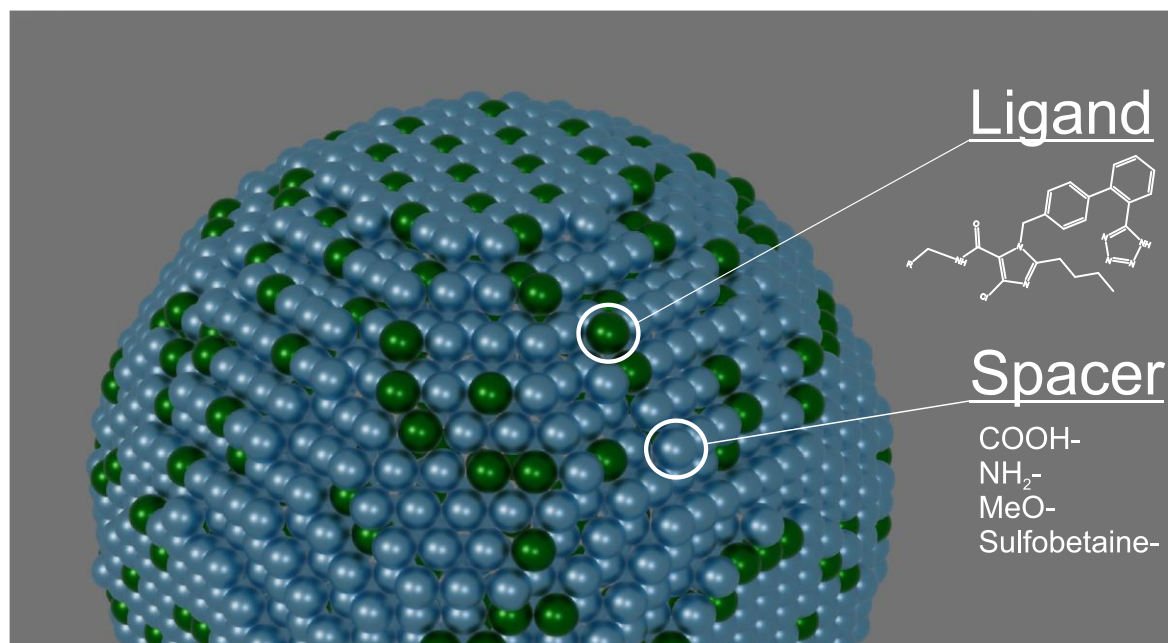
Evaluations of the Potential of the Nanoparticle Surface to Engage in Non-Specific Interactions

Manuscript

The experiments on the supported lipid bilayers were conducted with the support of T. Heißenberg and J. Gerber-Nolte, under the supervision and expert advice of Dr. I. Mey and Prof. C. Steinem (Institute of Organic and Biomolecular Chemistry, Research Group, Prof. Steinem, Georg-August-University Göttingen).

Abstract

In the design of nanoparticles as active drug delivery systems and diagnostics, the configuration of their surface significantly influences their interaction with biological systems. To modulate the nanoparticles' cell tropism, their surface is therefore frequently functionalized with target cell-specific ligands and replenished with non-functionalized spacer chains, which can carry charged end groups. The adjusted ligand valence should enable specific and consequently selective interactions with the target cells. However, the potential of the ligands and spacer chains to also enter non-specific interactions is often ignored. In the present study, the surface of the previously established model nanoparticles (Chapter 3), was critically assessed for its potential to engage non-specific interactions with biomembranes. The hydrophobicity and charge of both, the ligand and the spacer chains, were analyzed as potential influencing factors for non-specific adhesion. Therefore, binding experiments were conducted on target receptor positive cells and on supported lipid bilayers, which represent an established model system for biomembranes. Furthermore, the surface charge density of the model nanoparticles was calculated and correlated with the results obtained in the in vitro experiments.



Graphical Abstract

Introduction

When implementing active targeting concepts with nanoparticles (NPs) as the platform, their surface is functionalized with ligands to anchor them via specific ligand-receptor interactions on the surface of their target cells.^[1,2] To adjust the ligand valence on the NP surface, spacer chains can be used, which contribute to the spatial separation of the ligands. They furthermore enhance the stability of the NP dispersion due to steric repulsion.^[3,4] However, the initial contact between NPs and cell membranes is usually driven by the high surface energy of the NPs and thus non-specific.^[5] While specific binding is based on a unique combination of interactions between macromolecules, non-specific binding arises from a range of interactions between different atoms, molecules, and macromolecules. This includes e.g. van der Waals, electrostatic, hydration and hydrophobic forces.^[6,7] The intended specific interaction can even be surpassed by competing non-specific interactions between NPs and cell components.^[8] The potential of both ligands and spacers to engage in these non-specific interactions with cells often goes unnoticed.

The cell membrane of eukaryotic cells consists of partially charged phospholipids with embedded proteins and carbohydrates.^[9] Therefore, surface charge and hydrophobicity of NPs are two factors that may significantly influence the formation of non-specific interactions.^[10–12] NPs, which are composed of cationic polymers can directly interact with or even deposit into the negatively charged cell membrane, consequently inducing toxicity.^[9] Conversely, negatively charged NPs have been reported to engage in interactions with cationic sites on the plasma membrane.^[13] Furthermore, hydrophobic materials and ligands can attach non-specifically to the cell membrane, which consist of 50 % lipids.^[9] The extent to which the cell membrane even plays a decisive role in binding processes between ligands and receptors has been the subject of intensive research.^[14–16] Many lipophilic ligands are hypothesized to first undergo non-specific attachment to the cell membrane by 3D diffusion before being transported to their target receptor via 2D cell membrane diffusion.^[15] This two-step process of ligand-receptor binding, involving first non-specific attachment to the cell membrane, could also have impact on the binding behavior of NPs that are functionalized with lipophilic ligands. Additionally, charge and hydrophobicity of their surface promote the formation of a protein corona around the NPs, thus affecting their interaction with cell membranes indirectly.^[9,17] Protein adsorption increases with the surface charge density^[17] and alters the chemical identity of the NPs.^[5,18] Fleischer and Payne^[19] demonstrated that the surface charge of NPs can even dictate which cell membrane receptors the formed protein-nanoparticle aggregates bind to.

To achieve non-specific binding resistance, strategies must be implemented to lower the surface energy of the NPs.^[20] A widely used approach to mitigate protein adsorption and protect NPs against non-specific interactions is to coat them with polyethylene glycol (PEG).^[21,22] The observed “stealth” effect is contingent upon the density and conformation of the PEG chains on the surface of the NPs. PEG chains are amphiphilic with both hydrophobic and hydrophilic properties.^[23] Their antifouling capacity depends on the formation of a stable hydration layer around the NPs by hydrogen bonding with water molecules.^[24] Zwitterionic polymers are a frequently discussed alternative^[8,24,25] and are

even superior to PEG chains in terms of their antifouling properties.^[23,24] They are built of sequences of amines and acids or quarternized ammonium and various anionic groups, which classify them as sulfobetaines, carboxybetaines and phosphorylcholines; consequently, they exhibit both dipole moments and strongly charged groups.^[8,23] By forming strong interactions with water molecules through ionic solvation, they can effectively decrease the surface energy of NPs.^[23] Han et al.^[8] proved in their study an increased binding resistance for proteins and cells when the quantum dots under investigation were functionalized with a combination of zwitterionic polymers and nonionic oligoethylene glycol.

The aim of this study was to characterize the surface of well-established model NPs, specifically the ligand-functionalized polymer chains and the spacer chains in the shell, regarding their potential to engage in non-specific interactions with biomembranes. Thereby, the focus was on analyzing the effects of charge and hydrophobicity. The chosen model NPs were constructed as core-shell NPs built of a PLGA (poly(D,L-lactide-co-glycolide)) core and associated PLA-PEG (poly(ethylene glycol)-b-poly(D,L-lactide)) copolymers. The PEG chains extended outward as the shell. 25 % of the PEG chains were functionalized with EXP3174, an antagonist at the angiotensin-II type 1 receptor (AT1R), as ligand. The remaining PEG chains were designed as carboxy-terminated spacer chains, thus influencing the surface charge of the NP. This model NP was previously characterized regarding its specific interactions with target receptor positive rat mesangial cells (rMCs) (Chapter 3).^[26] In the present study the carboxy-terminated spacer chains were replaced by spacer chains of varying charge. The influence of their charge on the binding behavior of the NPs was analyzed. Additionally, the surface charge density of the NPs was calculated from zeta potential measurements. To decrease the surface energy of the NPs, the synthesis of a zwitterionic sulfobetaine head group for the spacer chains was established and assessed for its potential to affect the adhesion of the NPs to cell membranes. Furthermore, EXP3174, which is an active metabolite of losartan, was analyzed for its capacity to interact non-specifically with biomembranes in addition to its already verified specific binding at the AT1R (Figure 1). The ligand is characterized by its hydrophobicity.^[14] Various studies have shown that sartans interact strongly with the cell membrane and at least one interaction mechanism with their receptor occurs through diffusion across the cell membrane.^[16,27,28] In the investigated NPs, EXP3174 was coupled to the PEG chains via EDC/NHS chemistry. We hypothesized that this might lead to the formation of a reverse surfactant-like structure with the hydrophobic ligand as head group on the more hydrophilic PEG chain, well suited to interact non-specifically with biomembranes. Binding studies of the NPs were conducted on rMCs and on supported lipid bilayers composed of artificial lipids. The latter served as a model system to investigate the interaction of the NPs with isolated lipid bilayers without embedded proteins and carbohydrates. The present study therefore aims to enhance the understanding of the interaction potential of the NP surface with biomembranes. Furthermore, previously unnoticed interaction scenarios with regard to the reverse surfactant characteristics of ligand-functionalized polymer chains should be addressed.

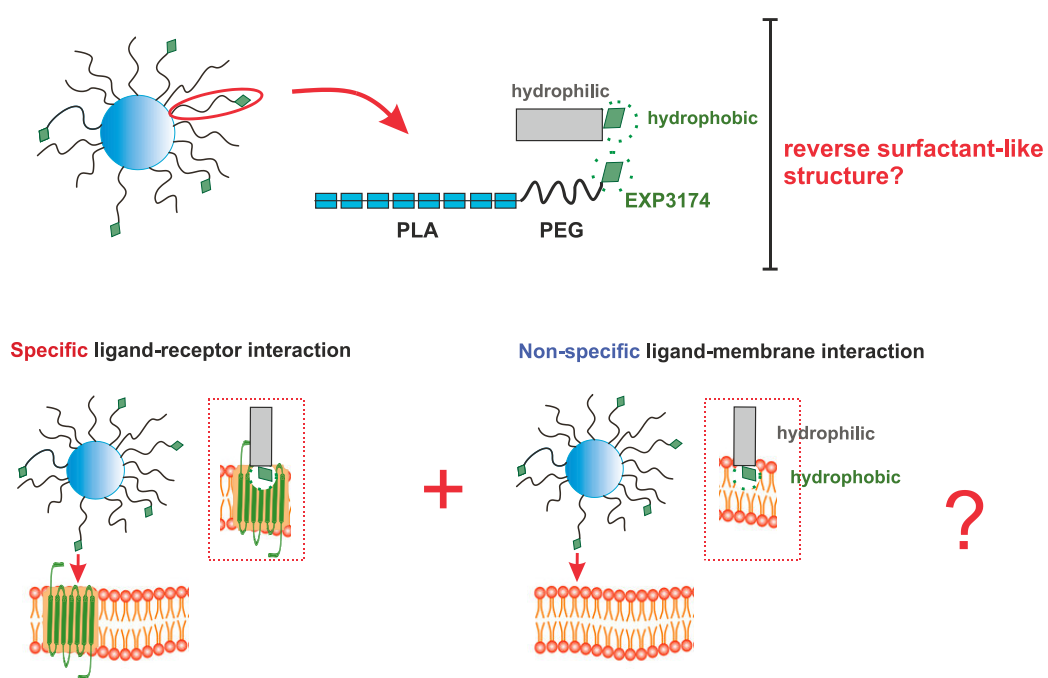


Figure 1 Graphical depiction of the research hypothesis: The reverse surfactant-like structure of EXP3174-functionalized PEG_{5K}-PLA_{10K} chains and their potential to engage in non-specific interactions with the lipid bilayer of the cell membrane in addition to the specific interactions with AT1R.

Results

Characterization of the Model NPs

The core-shell NPs chosen as a model in the present study were already well established in our group^[26,29] and composed of CY-5-labeled PLGA as core material associated with 25 % EXP3174-functionalized PEG_{5K}-PLA_{10K} chains and 75 % carboxy-terminated PEG_{5K}-PLA_{10K} spacer chains (COOH-PEG_{5K}-PLA_{10K}). The PEG chains form the shell. The NPs were previously characterized regarding their specific ligand-receptor interactions on target cells (Chapter 3).^[26] Due to the carboxy-terminated spacer chains, the NPs exhibited a negative zeta potential of -15.5 ± 7.4 mV (mean $\pm$ SD from five independent batches) (Table S1). Based on this NP design (25 %-EXP-NPs), the potential of the NP surface to engage in non-specific interactions was examined, by adjusting the charge and the ligand density in the shell.

The ratio of carboxy-terminated spacer chains relative to ligand-functionalized chains was varied between 0 % and 100 % to investigate their influence on the surface charge of the NPs (Figure 2A-B). With an increasing fraction of COOH-PEG_{5K}-PLA_{10K} chains, the hydrodynamic diameter of the NPs increased while their zeta potential decreased as expected. The surface charge density (σ) of the NPs was calculated according to equation 1 from the measured zeta potential (ξ) and the NP radius (a)^[30,31]:

$$\sigma = \frac{2\varepsilon_r\varepsilon_0\kappa kT}{ze} \sinh\left(\frac{ze\xi}{2kT}\right) \left[1 + \frac{1}{ka} \frac{2}{\cosh^2\left(\frac{ze\xi}{4kT}\right)} + \frac{1}{(ka)^2} \frac{8\ln\left[\cosh\left(\frac{ze\xi}{4kT}\right)\right]}{\sinh^2\left(\frac{ze\xi}{2kT}\right)} \right]^{\frac{1}{2}}$$

eq. 1

where ε_0 is the permittivity of the vacuum ($8.854 \cdot 10^{-12} \text{ C}^2 \text{ kg}^{-1} \text{ m}^{-3} \text{ s}^2$), ε_r is the dielectric constant of the medium ($78.5 \text{ C}^2 \text{ J}^{-1} \text{ m}^{-1}$), e is the elementary charge ($1.602 \cdot 10^{-19} \text{ C}$), k is the Boltzmann constant ($1.381 \cdot 10^{-23} \text{ J K}^{-1}$), and T is the absolute temperature (289.15 K) at which the measurement was conducted. The Debye-Hückel parameter (κ) was defined according to equation 2, where N_A is the Avogadro constant ($6.022 \cdot 10^{23} \text{ mol}^{-1}$):

$$\kappa = \left(\frac{2IN_A e^2}{\varepsilon_r \varepsilon_0 kT} \right)^{\frac{1}{2}}$$

eq. 2

The ionic strength of the electrolyte medium (10 mM NaCl solution) was determined using equation 3, with c_i being the molar concentration of the respective ion and z_i the charge number of the ion:

$$I = \frac{1}{2} \sum_i c_i z_i^2$$

eq. 3

The calculated surface charge density increased from $-0.13042 \text{ e}^- \text{ nm}^{-2}$ to $-0.25334 \text{ e}^- \text{ nm}^{-2}$, as the fraction of carboxy-terminated spacer chains in the shell of the NPs increased from 0 % to 100 % (Figure 2B). For NPs, which carry 100 % carboxy-terminated spacer chains on their surface (COOH-NPs), this corresponds to an average of one negatively charged moiety per 3.9 nm^2 NP surface area.

The surface area (S) occupied by a single PEG chain on the NP surface was calculated according to equation 4,^[32,33] where M_{PEG} is the molecular weight of a PEG chain (5000 g mol^{-1}), and f is the mass fraction of PEG in the PEG_{5K}-PLA_{10K} block copolymer blends. The density of the NPs (ρ_{NP}) was estimated to be 1.25 g cm^{-3} .^[34]

$$S = \frac{6 \cdot M_{PEG}}{dh \cdot N_A \cdot f \cdot \rho_{NP}}$$

eq. 4

An average surface area of 1.142 nm^2 per PEG chain was determined from the measured hydrodynamic diameters (dh) of the NPs (Table 1). These results indicated that less than every third PEG-chain contributed to the overall surface charge of the NPs.

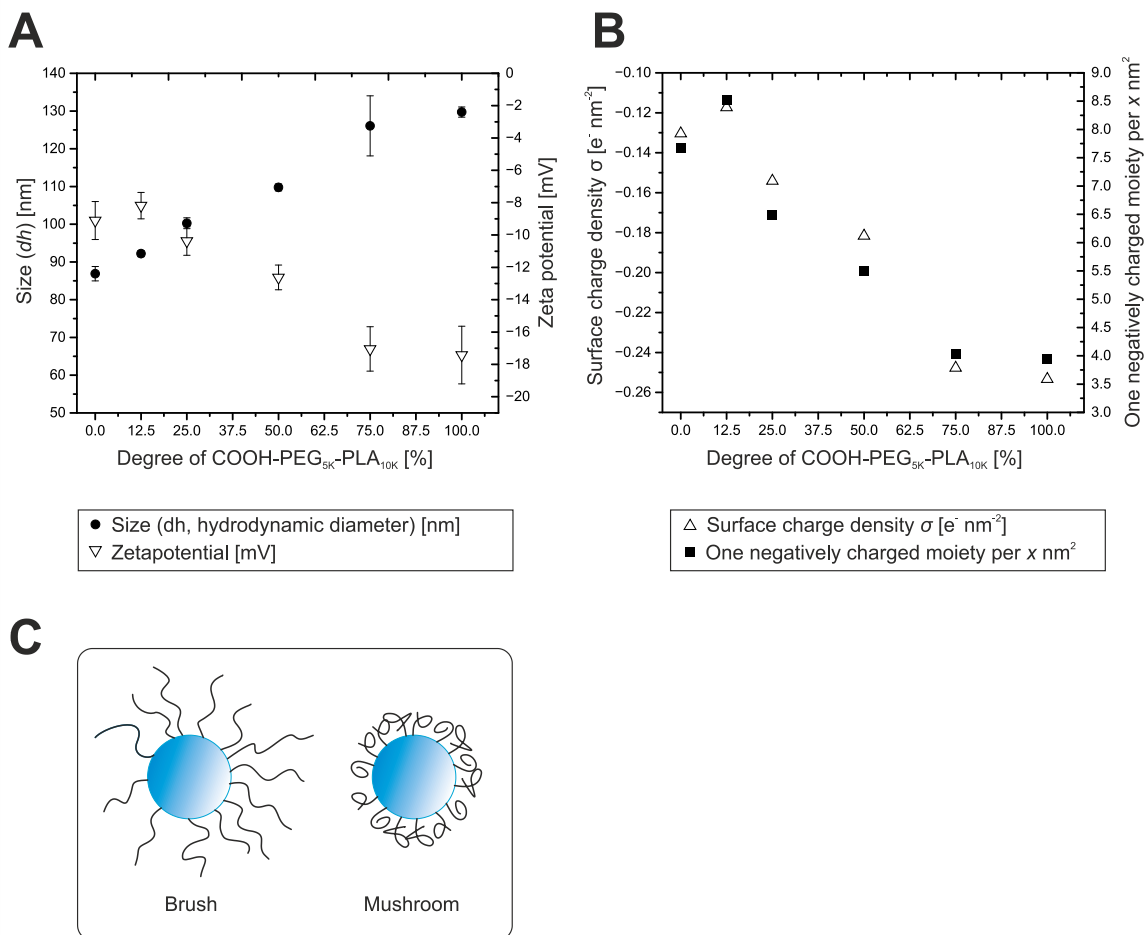


Figure 2 Characterization of the size (*dh*) and the charge of the NPs. (A) Size (*dh*) [nm] and zeta potential [mV] of the NPs measured using Malvern Zetasizer Nano ZS. Data are represented as mean $\pm$ SD (N = 3, n = 3). (B) The surface charge density σ [e⁻ nm⁻²] calculated according to equation 1 and the result converted to one negative charge per area [nm²]. (C) Illustration of brush and mushroom conformation of PEG chains.

The distance (*D*) between the PEG chains was calculated to an average value of 1.203 nm using equation 5.^[33]

$$D = 2 \sqrt{\frac{S}{\pi}}$$

eq. 5

Since *D* was smaller than the Flory radius (*R_F*) of the polymer, which was calculated according to equation 6 to be 5.989 nm, it could be assumed that the surface-grafted PEG chains adopted a brush conformation for all NP types (Table 1, Figure 2C).^[35]

$$R_F = a \cdot n^\nu$$

eq. 6

R_F , which corresponds to the radius of the semispherical volume of a PEG chain, is defined as a function of the number of monomer chains per polymer (n), the Flory constant (ν) and the length of one monomer (a).^[33,35]

Degree of COOH-terminated polymer chains [%]	NP size (dh) [nm]	Surface (S) that each PEG chain occupies on the NP [nm ²]	Distance (D) between two grafting sites [nm]
100	129.7	0.9486	1.099
75	126.1	0.9486	1.099
50	109.8	1.090	1.178
25	100.3	1.193	1.232
12.5	92.20	1.297	1.285
0	86.88	1.377	1.324
		Ø 1.142	Ø 1.203

Table 1 Representation of the NP size (dh) [nm], the calculated surface area occupied per PEG chain (S) and the calculated distance (D) between the grafting sites as a function of the degree of COOH-terminated polymer chains. The size was measured using Malvern Zetasizer Nano ZS. Data represented as mean ($N = 3$, $n = 3$).

Effects of Charge and Hydrophobicity on the Interaction with the Target Cells

In the previously established NP design (25 %-EXP-NPs), the carboxy-terminated spacer chains were replaced with spacer chains carrying differently charged head groups (carboxy-, amino-, methoxy-terminated) to examine the effect of charge on their adhesion to target cells. Furthermore, zwitterionic surfaces were generated by mixing amino- and carboxy-terminated spacer chains in equal proportions (referred to as mixed-charged spacer chains) and by synthesizing PEG_{5K}-PLA_{10K} chains carrying a sulfobetaine head group (referred to as zwitterionic spacer chains, ZW3-). The sulfobetaine head group was built up on amino-terminated block copolymers in two synthesis steps and subsequently characterized (Figure S1; Figure S2, Figure S3). Additionally, NPs with a ligand valence (50 % and 100 %) exceeding that required for receptor-mediated anchoring to the cell surface were produced,^[26,29] to investigate the effect of an increased ligand valence on the binding behavior of the NPs. All NPs were characterized regarding their hydrodynamic diameter (dh), polydispersity index (PDI) and zeta potential using DLS and NTA measurements (Table S1). The measured zeta potential values were in line with the surface modifications (Table S1).

Binding curves of the differently modified NPs were recorded on rMCs in serum-containing medium using flow cytometry (Figure 3A). As all NPs were functionalized with EXP3174, which induces uptake inhibition of the NPs in AT1R positive cells,^[36] the geometric mean of the cell-associated fluorescence (APC-A geo. Mean) was assessed as an indicator for the binding of NPs to the target cells surface. K_i values of the NPs could be derived from a classical saturation binding model,^[37,38] which in this case were not corrected for non-specific binding. They thus reflect specific and non-specific binding of the NPs toward their target cells. The determined K_i values were close to the concentration of 100 pM for all NP types (Table S2). Consequently, this NP concentration (100 pM) served as a basis for comparison between the data sets, when plotting fluorescence intensity histograms of cell-associated fluorescence (Figure 3B). Comparison of the means across all groups revealed a decrease in NP adhesion depending on their surface properties, following the order: NPs with increased ligand valence (50 %- or 100 %) > NPs with methoxy-terminated spacer chains > NPs with carboxy-terminated spacer chains > NPs with zwitterionic spacer chains > NPs with amino-terminated spacer chains > NPs with mixed-charged spacer chains.

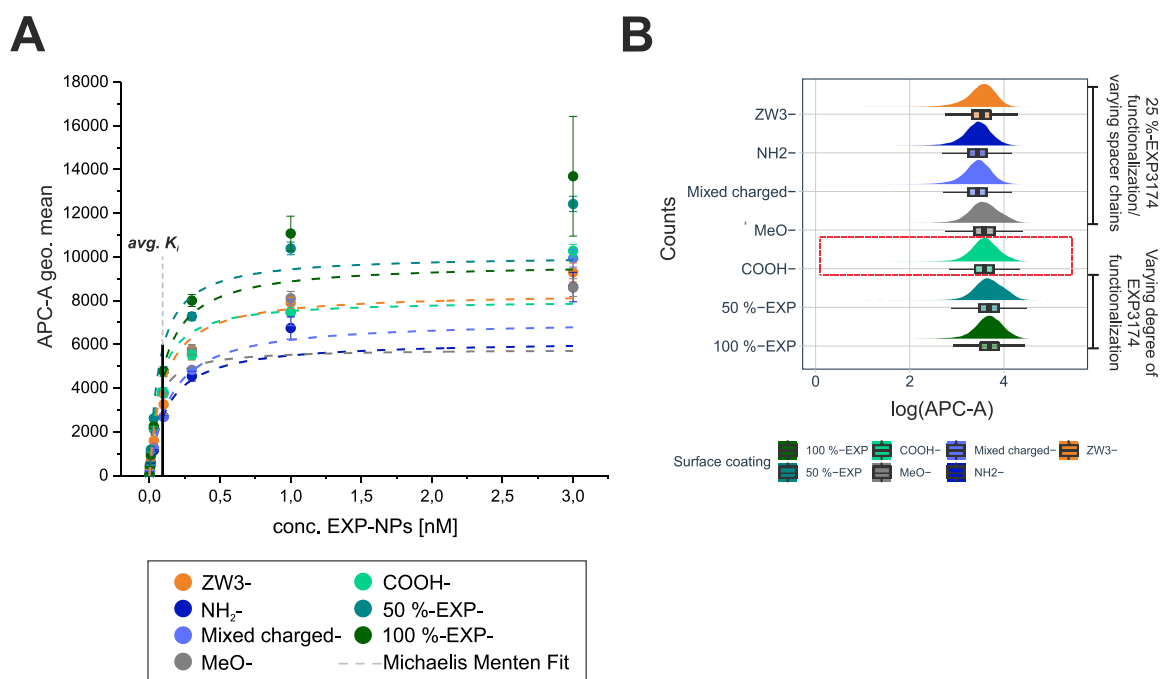


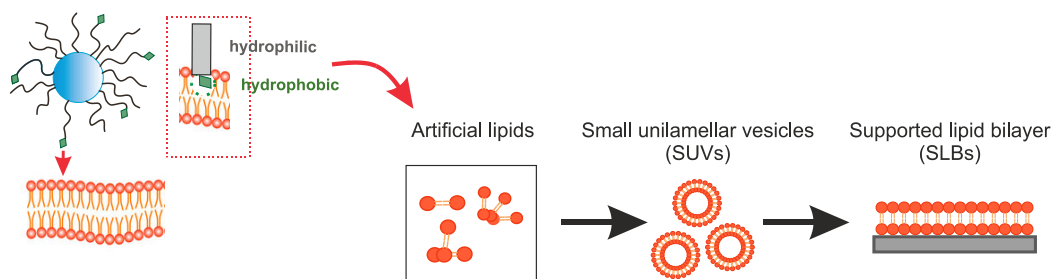
Figure 3 NP adhesion to rMCs. (A) Binding curves of NPs with distinct types of surface modifications recorded on rMCs using flow cytometry. The abbreviations (ZW3-, NH₂-, Mixed charged-, MeO-, COOH-) define the head group of the PEG_{5K}-PLA_{10K} spacer chains, while the 25 %-functionalization with EXP3174 was preserved. In the case of NPs with a deviating ligand valence (50 %-EXP-), COOH-PEG_{5K}-PLA_{10K} were preserved as spacer chains. The average K_i value of the NPs derived from a classical saturation binding model is highlighted as black line. (B) Fluorescence intensity histograms of cell-associated fluorescence after incubation with NP dilutions at a concentration equal to their average K_i value (100 pM). The data was subjected to a Welch-ANOVA (p -value < $2.2 \cdot 10^{-16}$) with subsequent Bonferroni corrected post hoc paired t-test, as the Levene test indicated variance heterogeneity ($\text{Pr}(>F) = 2.2 \cdot 10^{-16}$). The effect size was evaluated according to Cohen^[39] and was indicated as negligible or small or moderate depending on the compared groups. (Please refer to the Statistics section in Material and Methods for further detail.)

The effect of the distinct surface modifications was statistically examined using Welch-ANOVA (p -value $< 2.2 \cdot 10^{-16}$) with subsequent Bonferroni corrected post hoc paired t-test, as the Levene test indicated variance heterogeneity ($\text{Pr}(>F) = 2.2 \cdot 10^{-16}$). Subsequently, the effect size was evaluated according to Cohen.^[39] The Welch-ANOVA indicated statistically significant differences for all NP types except for two groups, namely NPs with amino-terminated spacer chains and NPs with mixed-charged spacer chains. However, the effect size^[39] between the groups could only be classified as negligible or small or moderate. The statistically significant difference between the groups indicated by the Welch-ANOVA was therefore possibly due of the size of the data sets examined (> 28000 data points). Consequently, the biological relevance of the results needs to be scrutinized.

Investigation of the Interaction between Ligand-Functionalized NPs and Lipid Bilayers

To investigate the interaction potential of EXP3174-functionalized PEG chains with isolated lipid bilayers (Figure 1), experiments on supported lipid bilayers (SLBs) were performed (Figure 4). By selecting SLBs as a model system, non-specific interactions caused by other membrane components (e.g. carbohydrates and proteins) could be eliminated from the analysis. The adhesion of 25 %-EXP-NPs to the SLBs was compared with that of COOH-NPs, which served as a control. First, to analyze the adhesion of the NPs qualitatively, CLSM experiments were conducted on ATTO 488 stained SLBs. Therefore, small unilamellar vesicles (SUVs) were prepared either from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), a zwitterionic phospholipid, or a mixture of POPC and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS), a negatively charged phospholipid, or each with added cholesterol and spread on silicon wafers to form SLBs (Figure 4).

A Non-specific ligand-membrane interaction



B

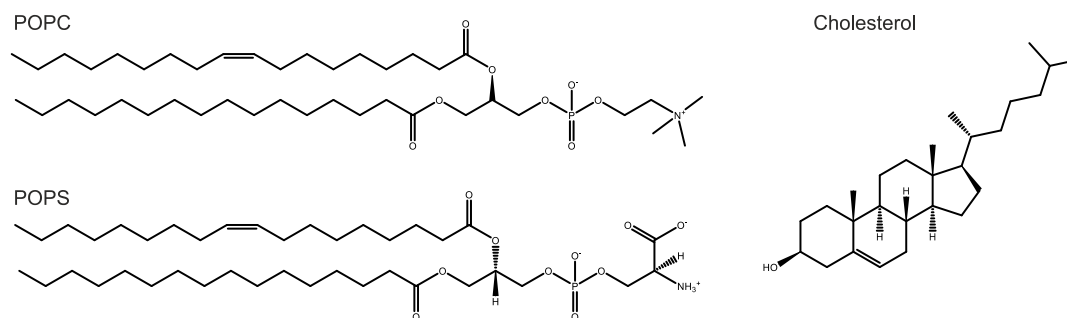


Table B

<i>Lipid films</i>	<i>Net charge</i>
POPC	neutral
POPC + 20 % POPS	negative
POPC + 30 mol% Cholesterol	neutral
POPC + 20 % POPS + 30 mol% Cholesterol	negative

Figure 4 Graphical depiction of the research hypothesis and the preparation of SLBs as a model system for its examination. (A) Schematic depiction of the preparation of supported lipid bilayers (SLBs) using SUVs. (B) Chemical structures of the components used for the preparation of the SLBs and representation of their net charge in Table B.

CLSM images and Z-stacks were recorded after application of the NPs (Figure 5, Figure 6, Figure S4, Figure S5). The 3D images and orthogonal views revealed that part of the NPs had deposited on the bilayers, while the rest was dispersed in the fluid above the SLBs. There were no apparent differences between the EXP3174-functionalized NPs and the control NPs regarding their adhesion behavior to the SLBs.

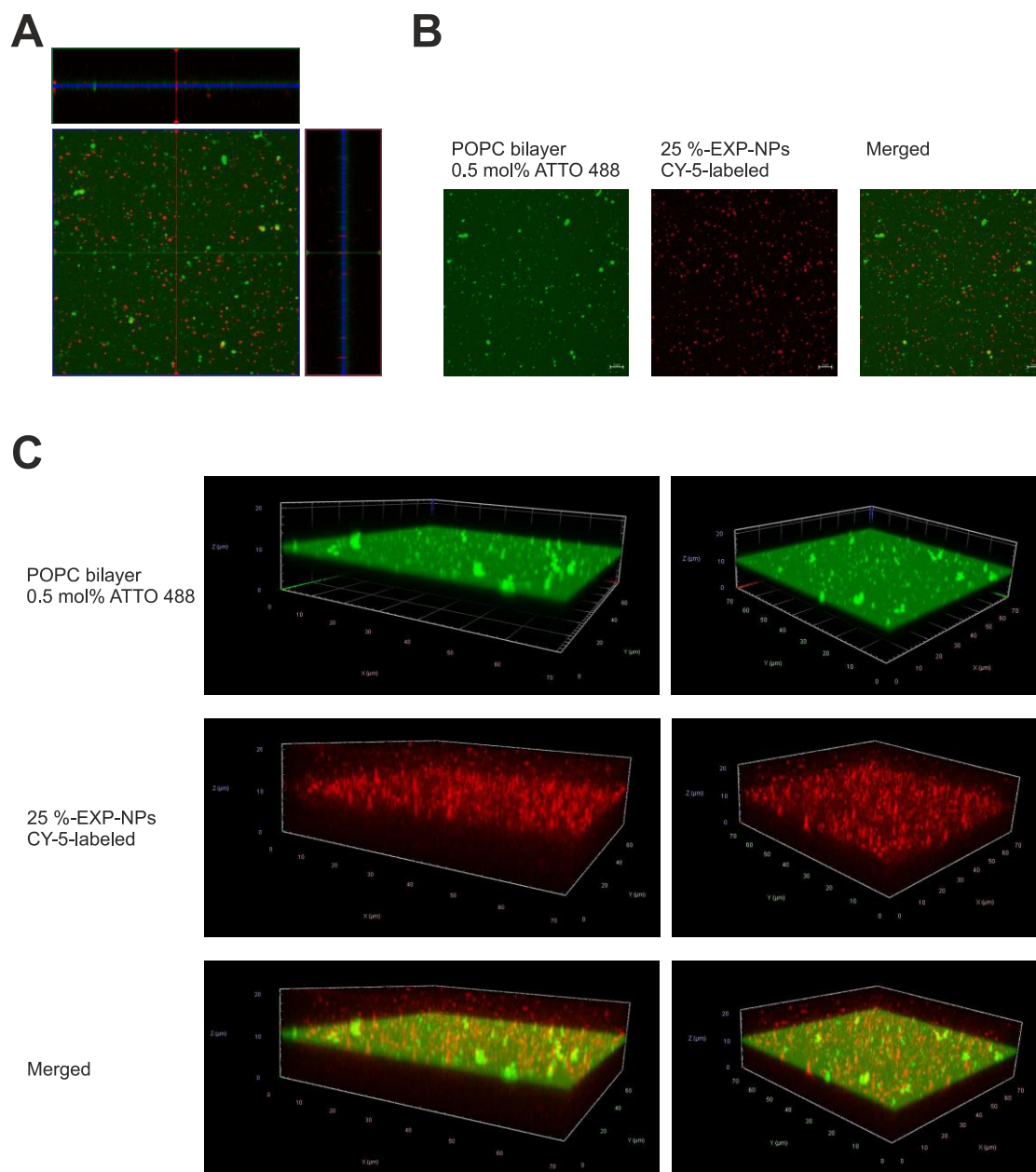


Figure 5 Localization of 25 %-EXP-NPs relative to the POPC lipid bilayer. The lipid bilayer was stained with 0.5 mol% ATTO 488 (green) and the NPs were labeled with CY-5 (red). (A) Orthogonal view of the NP distribution. (B) Images of the split channels of the bilayer and the NPs, and image of the merged channels (scale bar: 5 μ m). (C) 3D images of the membrane, the NP distribution, and the merged channel.

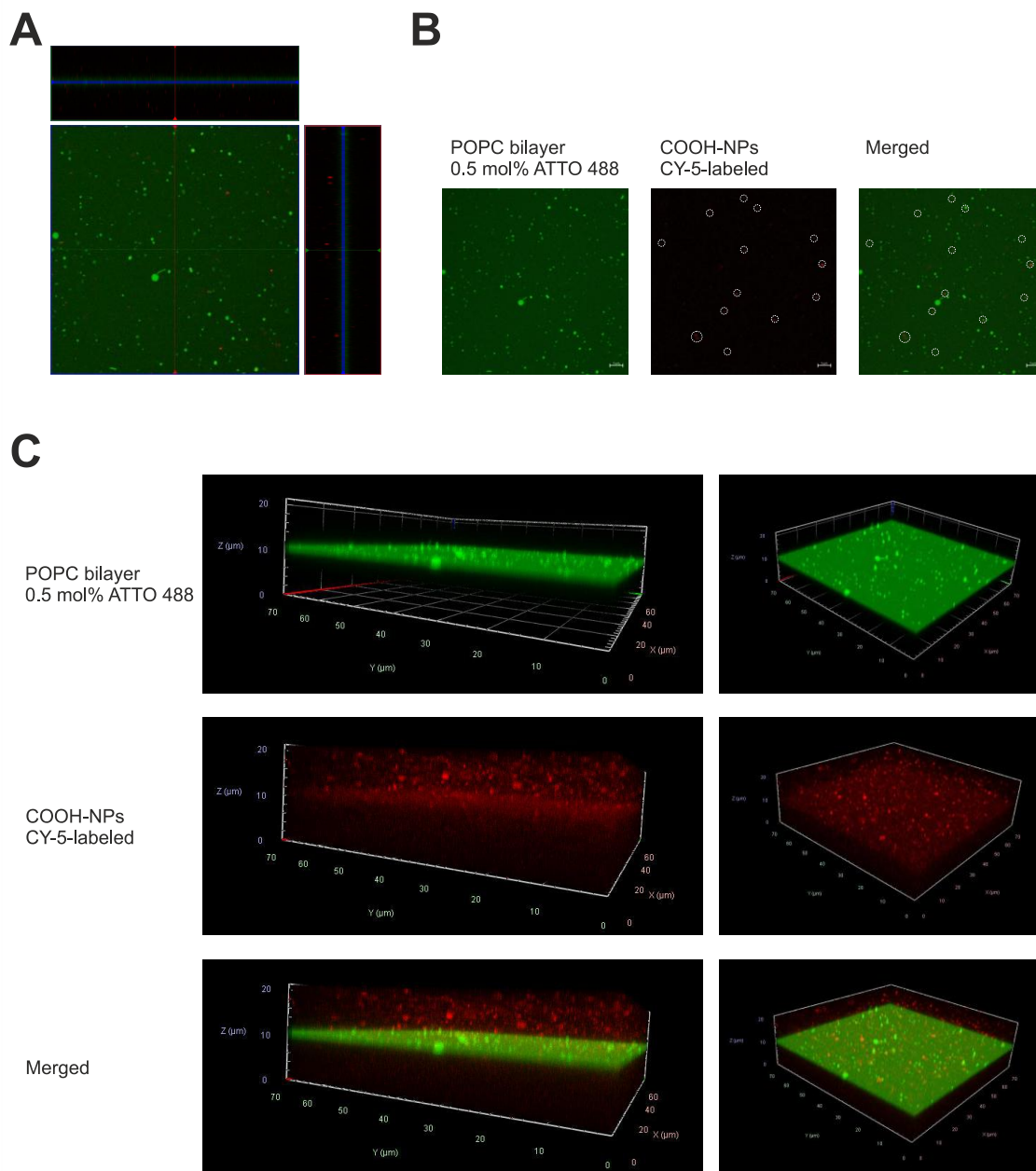


Figure 6 Localization of COOH-NPs relative to the POPC lipid bilayer. The lipid bilayer was stained with 0.5 mol% ATTO 488 (green) and the NPs were labeled with CY-5 (red). (A) Orthogonal view of the NP distribution. (B) Images of the split channels of the bilayer and the NPs, and image of the merged channels (scale bar: 5 μm). NP clusters are delineated by white circular markers. (C) 3D images of the membrane, the NP distribution, and the merged channel.

For a quantitative analysis, RfS measurements were conducted on POPC and POPC/POPS bilayers to determine variations in layer thickness following the application of NPs. First, open circuit - flow measurements were performed. The formation of lipid bilayers could be clearly determined by an increase in the optical thickness (Δ) of 6 – 7 nm when the SUV emulsions were rinsed over the silicon wafers. After the bilayers had formed with a stable layer thickness on the wafers, the NP samples were applied. However, no further increase in layer thickness on the lipid bilayers could be observed

(Figure 7). To test the incubation time of the NPs on the bilayers as an influencing factor for their adhesion, closed circuit - flow measurements and measurements under static conditions were conducted on POPC membranes (Figure S6). Under both measurement conditions, the bilayers were incubated with the NPs for at least 1 h, either under flow or static conditions. However, no adhesion for 25 %-EXP-NPs and COOH-NPs to the bilayers could be detected.

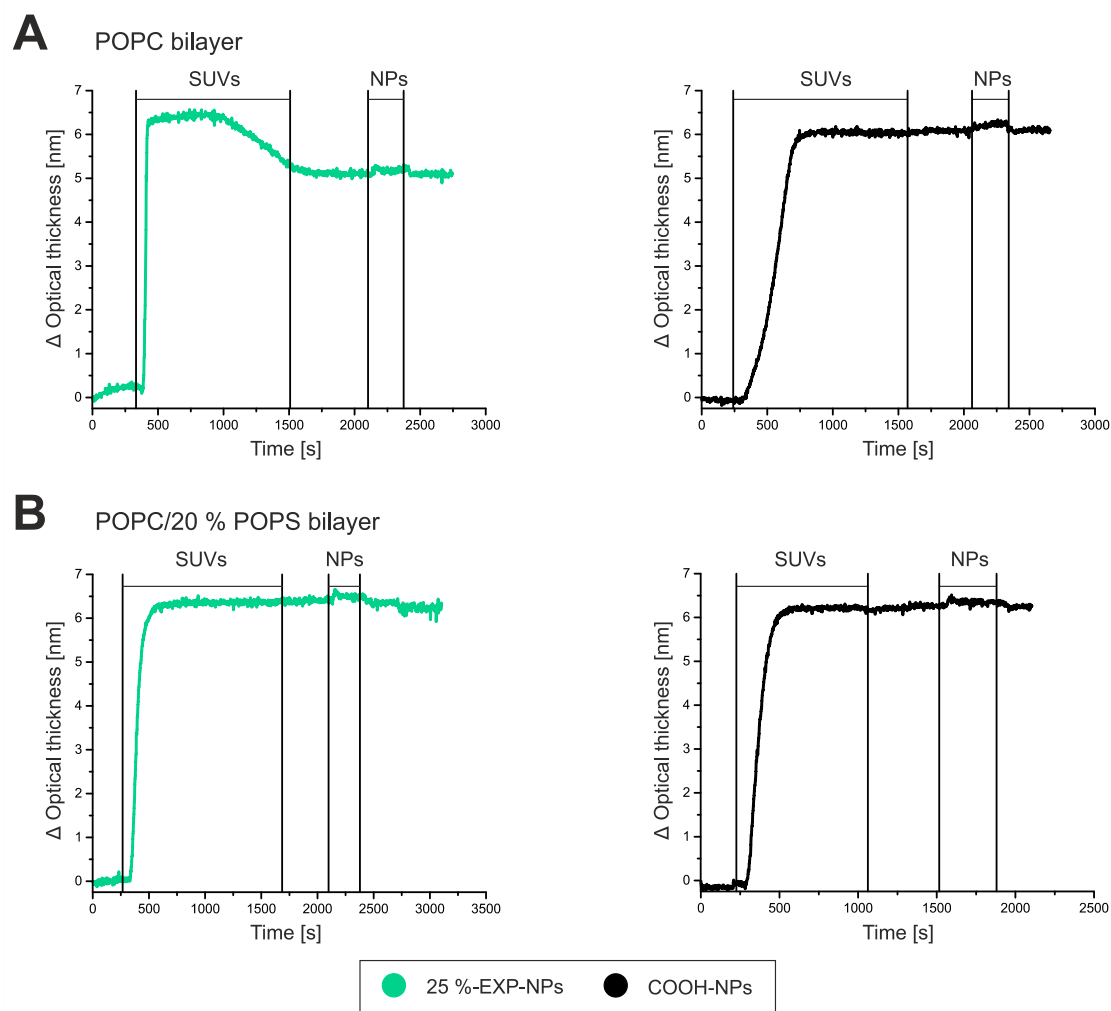


Figure 7 RlfS experiments on lipid bilayers under open circuit - flow measurement conditions. The NPs (25 %-EXP-NPs or COOH-NPs) were applied to the system, rinsed over the bilayer, and immediately discarded. (A) RlfS measurements on bilayers composed of POPC. (B) RlfS measurements on bilayers composed of POPC/20 % POPS.

Discussion and Conclusion

In the present study, EXP3174-functionalized model NPs with carboxy-terminated spacer chains in their shell were employed to examine the potential of the lipophilic ligand and the charged spacer chains to exhibit non-specific interactions with biomembranes. By varying the percentage of carboxy-terminated spacer chains in the NP design, it was successfully demonstrated that the surface charge density increased as a function of the number of carboxylic acid groups in the NP shell. However, our analysis indicated that even NPs, which were composed of 100 % carboxy-terminated spacer chains in their shell (COOH-NPs), carried only one negatively charged moiety per 3.9 nm² surface area. The comparison with the calculated average surface area occupied by a single PEG chain (1.142 nm²) revealed that less than every third chain thus contributed to the negative surface charge of the NPs. However, considering the cross-sectional area of a stearic acid of 0.22 nm² as a reference, it follows that each carboxy-terminated PEG chain could theoretically have contributed to the surface charge.^[40] This raises questions, as the carboxylic acid end groups of the PEG chains should have been quantitatively deprotonated at the pH value (pH = 7.05) at which the experiments were conducted. Furthermore, it was shown that the distance between the grafting sites was smaller than their Flory Radius (R_F). The polymer chains should therefore adopt a brush conformation.^[35] In this case, the negatively charged head groups of the polymer chains were expected to be presented toward the surface of the NPs and to quantitatively contribute to the surface charge of the NPs. A possible explanation could be that the degree of deprotonation of the carboxylic acid groups is influenced by the local environment surrounding the NPs, which differs from that of the bulk solvent.^[41] Moreover, Damodaran et al.^[42] showed in their study using X-ray photoelectron spectroscopy that the formation of a brush conformation is also dependent on the PEG density (t) on the NP surface. They found that the brush conformation only fully develops when the surface concentration of PEG chains is high enough to form a packed and dense molecular layer. In addition to the necessary condition $D < R_F$, they introduced $t > 2R_F$ as a second sufficient condition.^[42] Their experiments furthermore indicated that the composition of the matrix, in case of the NPs under consideration their PLGA/PLA core, has a great impact on the conformational properties of the PEG chains. Taking these additional conditions into account, it can no longer be conclusively assumed that the surface-grafted PEG chains quantitatively adopted brush conformation in the present model NPs.

By replacing the carboxy-terminated spacer chains with chains carrying differently charged head groups, the influence of charge on the NPs' binding behavior toward target cells was examined. It is widely described in the literature that charged moieties foster interactions of NPs toward biomembranes: Positively charged groups on the surface of NPs promote their interaction with the negatively charged cell membrane^[9] and the interaction of negatively charged NPs is explained by their clustering on cationic isles.^[13] Conversely, neutral surface charge should prevent unintended interactions between NPs and cell surfaces.^[13] Moreover, adhesive effects can be achieved by employing zwitterionic structures to lower the surface energy of NPs.^[8,20] This gives rise to the

expectation that the charged end groups of spacer chains could enhance the adhesion of NPs through non-specific electrostatic interactions, in addition to the NPs' specific ligand-mediated interaction. The anticipated charge-mediated effect is expected to follow this order: amino-terminated or carboxy-terminated spacer chains > methoxy-terminated spacer chains > spacer chains carrying zwitterionic head groups. The findings of our study, seem to deviate from this expectation. However, the biological relevance of the results must be critically questioned. The statistical analysis revealed significant differences in the binding of NPs with varying surface charges toward target cells. Nevertheless, the Cohen's effect size was classified as negligible, small or moderate.^[39] The obtained results indicate that other effects outweigh the influence of the surface charge in the present model system. The exemplary calculation of the surface charge density on the NPs' surface raises the question of whether one charged group per chains is sufficient to significantly influence the binding behavior of the NPs. In contrast, the zwitterionic spacers described in the literature are usually polyampholytes with varying sequences and ratios of charge.^[25,43–45] The geometric congruence between NPs and the cell membrane, particularly evident in the binding of NPs in clathrin-coated pits and the resulting increase in the number of possible specific ligand-receptor interactions,^[26,37] could further reduce the influence of charged spacer chains on the binding of the NPs. So far, the interpretation of the results has assumed that the surface of the NPs appeared pristine and bare when interacting with the target cell surface. However, in serum-containing environment, proteins and lipids accumulate around the NPs to form a protein corona.^[5,19,46] The formation of a protein corona decreases the surface energy of the NPs and thus affects their adhesion toward cell surfaces.^[46] Unfortunately, these effects, along with the influence of NPs' surface charge on the formation of the protein corona, could not be examined within the scope of the present study.

Due to the hydrophobicity of EXP3174 and its reported interaction with the target receptor by 2D membrane diffusion,^[14–16] we proposed the hypothesis that the ligand not only engages in specific interactions with the receptor but also contributes to non-specific interactions of the NPs with the cell membrane. To investigate this hypothesis, first binding studies of NPs with increased degree of EXP3174-functionalization, exceeding that required for receptor-mediated anchoring,^[26,29] were performed on receptor-positive target cells. Due to the lack of conclusive results from these studies, further binding experiments were conducted on SLBs. SLBs have the advantage of a well-controlled model system that has already been used in several studies to investigate the interaction of NPs with biomembranes.^[47,48] The selection of POPC and POPS as lipid components of the SLBs was intended to investigate the influence of the surface charge of cell membranes^[47] on the binding behavior of the NPs. The addition of cholesterol served to modulate the fluidity of the membranes.^[49] However, it should be considered that these model systems are limited by the fact that the lipid composition can only partially cover that of the cell membrane.^[47] Furthermore, the interaction with the wafers influences the properties (e.g. bending rigidity) of the lipid bilayers.^[5,50] The recorded CLSM images showed that the implementation of EXP3174 into the NP surface did not affect their adhesion to the SLBs compared to NPs without ligand-functionalization, independently on the membranes surface charge and composition. However, statistically significant results from CLSM images could only be achieved with a substantially higher sample volume. What is particularly

Chapter 4

unexpected, however, is that although the CLSM images indicated that part of the NPs had deposited on the bilayers, no adhesion of the NPs to the SLBs could be detected in the quantitative RfS measurements, even when the incubation time was increased up to 1 h. In a previous study published by Jing and Zhu,^[48] it was shown that the adsorption equilibrium of NPs from dispersion onto SLBs was reached within 2 min. The authors employed quartz crystal microbalance (QCM) measurements to determine the adhesion of the NPs. A more detailed review of the literature indicates that conventional RfS may not have sufficient sensitivity to quantify the adhesion of NPs on the lipid bilayers.^[51] In comparable studies, the technique was therefore only used in combination with QCM^[52] or as polarization reflectometric interference spectroscopy (PRfS).^[51,53] This clearly weakens the significance of our results.

While the focus of the present work was on the characterization of the NPs' surface, other studies have shown that the core material of NPs also plays a decisive role for engaging in non-specific interactions.^[31,54] Non-specific adhesion behavior consequently seems to be a holistic challenge in the design of NPs. The present study clearly highlights that each NP system must be assessed individually for its potential to engage in non-specific interactions with cells and that some effects remain only partially characterized and are yet to be fully understood.

Material and Methods

Materials

All materials and reagents were purchased from Sigma Aldrich (Taufkirchen, Germany or St. Louis, USA) unless otherwise stated. Dulbecco's phosphate-buffered saline (DPBS) was acquired from Gibco™ (Life Technologies, Paisly, UK), and Leibovitz's L-15 medium was purchased as medium without phenol red from Gibco™ (Life Technologies, New York, USA). The ultrapure water used for the experiments was treated with a Milli-Q EQ 7000 system (Merck, Darmstadt, Germany) equipped with Milli-Q Biopak filter (Merck, Darmstadt, Germany) and freshly taken every day. It is referred to as Milli-Q water in the following.

Cell Culture

Rat mesangial cells (rMCs) were kindly gifted by Prof. Armin Kurtz (Institute of Physiology, University of Regensburg, Regensburg, Germany). The cell line was chosen as a model cell line for the cell surface interaction studies due to the stable expression of the target receptor, AT1R (angiotensin-II type 1 receptor), as previously shown by our group.^[55] The cells were cultured in RPMI 1640 medium containing 10 % fetal bovine serum (FBS) (South America Origin, Pan-Biotech, Aidenbach, Germany, Lot-No.: P201004) supplemented with insulin-transferrin-selenium (ITS) (Gibco, Life Technologies, New York, USA) and 100 nM hydrocortisone in a water saturated, carbon dioxide enriched (5 % CO₂) atmosphere at 37 °C.

Block Copolymer Synthesis and Characterization

COOH-PEG_{5K}-PLA_{10K}, Boc-NH-PEG_{5K}-PLA_{10K}, and MeO-PEG_{5K}-PLA_{10K} block copolymers were synthesized via ring-opening polymerization of the cyclic lactone 3,6-dimethyl-1,4-dioxane-2,5-dione with the organocatalyst 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) at RT. COOH-PEG_{5K}-OH and tBoc-NH-PEG_{5K}-OH (Jenkem Technology, Plano, USA, M_n 5000 ± 500 g mol⁻¹, as stated on certificate of analysis) and MeO-PEG_{5K}-OH (M_n 5000 g mol⁻¹) were used as macroinitiators for the reaction.^[56,57] In brief, polyethylene glycol (PEG) macroinitiators (1 equiv.) were reacted with 3,6-dimethyl-1,4-dioxane-2,5-dione (70 equiv.) and DBU (3.5 equiv.) in dichloromethane (DCM) as solvent at room temperature (RT). The reaction was quenched with benzoic acid in excess (17.5 equiv.) after 1 h. The resulting block copolymers were precipitated in ice-cold diethyl ether, filtered, and dried under the nitrogen flow. In case of tert-butyloxycarbonyl (tBoc)-protected block copolymers, the protecting group was subsequently removed by addition of trifluoroacetic acid (TFA). Identity and purity of the block copolymers were verified by ¹H-NMR spectra recorded on a Bruker Avance III HD 400 operating at 400 MHz; the block copolymers were dissolved in deuterated chloroform (CDCl₃).^[58] Furthermore, the molecular weight of the synthesized polymers was

determined from the ^1H -NMR spectra as previously described by our group.^[37] Topspin 4.1.4 software was used to evaluate the NMR spectra (Chapter 3, Figure S2).

The zwitterionic head group (sulfobetaine) was built up in two reaction steps on the deprotected amino-terminated polymer. *(Please refer to the Supplementary Information for further information on the sulfobetaine head group synthesis and characterization (Figure S1, Figure S2, Figure S3).)*

Fluorescence Labeling of PLGA with CY-5

Poly(D,L-lactide-co-glycolide) (PLGA) (Resomer RG 502, carboxylic acid-terminated, M_w 7000-17000, Sigma-Aldrich, St. Louis, USA) was fluorescently labeled by covalent linkage with cyanine-5-amine (Lumiprobe GmbH (Europe), Hannover, Germany). In brief, carboxylic acid-terminated PLGA (1 equiv.) was dissolved together with cyanine-5-amine (CY-5) (0.1 equiv.) in dry *N,N*-dimethylformamide (DMF). The carboxylic acid group was activated by the addition of (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (2 equiv.) and DIPEA (4 equiv.) and the reaction mixture was stirred overnight at RT under light protection. To remove the excess of free dye, the polymer was precipitated several times in ice-cold diethyl ether until the supernatant was no longer blue. The product was dried under the nitrogen flow and characterized by FTIR. FTIR spectra were recorded on an Agilent Technologies Cary 630 FTIR (Agilent, Santa Clara, USA) with 32 scans, 8 cm^{-1} resolution (Chapter 3, Figure S3).

Losartan Carboxylic Acid (EXP3174) Synthesis and Characterization

Losartan carboxylic acid (EXP3174) was obtained by oxidation of losartan with potassium permanganate as previously described by our group.^[59] Therefore, losartan potassium (Santa Cruz Biotechnology, Heidelberg, Germany) was diluted in 0.1 M sodium hydroxide solution (NaOH) and cooled down to $0\text{ }^\circ\text{C}$. An ice-cold, aqueous solution (0.2 M) of potassium permanganate (Merck, Heidelberg, Germany) was added dropwise in a 2.3-fold excess concentration. The reaction mixture was stirred for 4.5 h at RT. The product was then precipitated by adjusting the pH to acidic conditions with 1 M hydrochloric acid (HCl). Subsequently, the precipitate was washed twice with ice-cold water (3000 g, $4\text{ }^\circ\text{C}$, 15 min). The product was isolated by adjusting the pH to moderately alkaline conditions with 1 M NaOH. Remaining precipitated manganese dioxide was removed by centrifugation (3000 g, $4\text{ }^\circ\text{C}$, 15 min). The product was precipitated from the supernatant by acidification with 1 M HCl and then extracted with ethyl acetate in the separating funnel. The solvent was removed under rotation, the product was taken up in ethanol, precipitated in an excess of water and then freeze-dried (Alpha 2-4 LSCplus, Christ, Osterode am Harz, Germany) to obtain a dry powder. Identity and purity of EXP3174 were verified by ^1H -NMR and high-resolution mass spectrometry analyses. The ^1H -NMR spectrum was recorded on a Bruker Avance III HD 400 operating at 400 MHz. The product was therefore diluted in deuterated dimethyl sulphoxide ($\text{DMSO-}d_6$). Mass spectrometry analyses were performed on a Q-TOF 6540 ultrahigh definition (Agilent

Technologies, Waldbronn, Germany) using an electrospray ionization source (ESI) (Chapter 3, Figure S4).

EXP3174 Coupling

For EXP3174-PEG_{5K}-PLA_{10K}, deprotected amino-terminated block copolymer was functionalized with EXP3174 using EDC/NHS coupling chemistry. The ligand was coupled via its carboxylic acid functionality to the polymer. To activate the carboxylic function, EXP3174 (3.5 equiv.), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (10 equiv.) and *N*-hydroxysuccinimide (NHS) (5 equiv.) were dissolved in dry DMF. Subsequently, a solution of NH₂-PEG_{5K}-PLA_{10K} (1 equiv.) and *N,N*-diisopropylethylamine (DIPEA) (17.5 equiv.) in DMF was added dropwise to the ligand solution. The reaction mixture was stirred for 24 h at RT. For purification, the functionalized polymer was precipitated in a mixture of methanol/diethyl ether (1:15, v/v). The resulting product was dialyzed (using SpectraPor 6-8 kDa molecular weight cut-off, RC membrane, Spectrum Laboratories, Inc, Los Angeles County, USA) against ethanol, 100 mM borate buffer (pH 8.5) and Milli-Q water (1:1:8, v/v) for 24 h followed by Milli-Q water for 12 h. The coupling efficiency was determined by detecting free, unreacted amino groups via Fluram assay (Chapter 3, Figure S5).

Fluram Assay

The detection of free amino groups was carried out using the Fluram assay adapted from Udenfriend et al.^[60] Briefly, 10 µL of the respective test solution (sample, positive or negative control) were added to a white 96-well plate (Greiner Bio One, Frickenhausen, Germany) with 170 µL of borate buffer (pH 8.5, 50 mM) and shaken (150 rpm) for 5 min. Afterwards, 20 µL of a solution of 4'-phenylspiro[2-benzofuran-3,2'-furan]-1,3'-dione, fluorescamine (fluram) in acetone (0.3 mg mL⁻¹) were added and incubated together with the test solutions at RT for 15 min under shaking (150 rpm). The fluorescence was measured on a FLUOstar Omega plate-reader (BMG Labtech, Ortenberg, Germany) with an excitation wavelength of λ 380 nm. The emission was recorded on a wavelength of λ 485 nm (Chapter 3, Figure 5).

Nanoparticle Preparation

Core-shell nanoparticles (NPs) were obtained by nanoprecipitation method.^[61] For this purpose, the synthesized block-copolymers were adjusted together with CY-5-labeled PLGA (CY-5-PLGA) in acetonitrile (ACN) to a concentration of 10 mg mL⁻¹. The ratio of CY-5-PLGA core to block-copolymer shell was 3:7 (m/m). To achieve the desired degree of surface functionalization of the nanoparticles, the appropriate amount of EXP3174-terminated polymer within the block-copolymers was utilized. The organic phase was added dropwise into the 10-fold excess of stirring (930 rpm) aqueous phase (10 % DPBS (v/v) in Milli-Q water) and stirred for 3 h to remove the ACN. Subsequently, the volume

was concentrated by centrifugation at 3000 g for 15 min using Microseps Advance 30K centrifugal filters (molecular weight cut-off 30 kDa, Pall Corporation, New York, USA).

Nanoparticle Characterization

Dynamic light scattering (DLS)-based measurements (particle size distribution, polydispersity index, zeta potential) were carried out with a Malvern Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany) equipped with a 633 nm He-Ne laser operating at an angle of 173°. The software used for data collecting and processing was Zetasizer software version 7.12 (Malvern Instruments, UK). To determine the size distribution, the concentrated samples were diluted 1:20 with 10 % DPBS in Milli-Q water (10 % DPBS (v/v)). 90 µL sample volume were measured in micro-UV-cuvettes (Carl Roth, Karlsruhe, Germany) at a controlled temperature of 25 °C (120 sec equilibration time). Three repetitions with 11 runs of 10 sec duration were performed for each sample. The data was analyzed with the software-integrated analysis model for “general purposes (normal resolution)”. For zeta potential measurements the samples were diluted 1:200 with a 10 mM NaCl solution (pH = 7.05). The samples were measured in folded capillary zeta cells (Malvern, Herrenberg, Germany) at 25 °C. Three repetitions with at least 60 runs were performed for each sample. The data was analyzed by “auto mode”.

Furthermore, average size (hydrodynamic diameter), size distribution and concentration [particles mL⁻¹] of the NPs were determined using nanoparticle tracking analysis (NanoSight NS300, Malvern, Worcestershire, UK). The device was equipped with an O-Ring top-plate and the data was analyzed with NanoSight NTA 3.4 software (NTA Malvern Panalytical). Before measurement, all samples were diluted with Milli-Q water to achieve a good particle per frame value (20-100 particles/frame). The samples were applied by manual injection with sterile syringes. Each sample was measured in three cycles of 60 sec duration (screen gain: 1.0; camera level: 16) at RT. The detection threshold was set at 3.

Flow Cytometry

RMCs were seeded in TC-treated 24-well plates (Costar, Corning Incorporated, New York, USA) at a density of 60,000 cells/well and cultivated for 24 h (37 °C, 5 % CO₂), before proceeding for the flow cytometry analysis. On the day of experiment, the cells were at least 90 % confluent. To determine nanoparticle binding curves, serial dilutions (3 nM to 3 pM) of EXP3174-functionalized nanoparticles produced with CY5-labeled PLGA were prepared in Leibovitz's L-15 medium. The cells were set up for the experiments by discarding RPMI 1640 medium. They were then incubated with 200 µL of each nanoparticle dilution (3 replicates) under gentle shaking (50 rpm) for 30 min (37 °C, 5 % CO₂). After the incubation period, the nanoparticle dilutions were discarded, and the cells were washed once on-plate with 500 µL DPBS. For harvesting, 300 µL of trypsin (PAN-Biotech, Aidenbach, Germany) were added and the cells were incubated for 2 min (37 °C, 5 % CO₂). After cell detachment, 500 µL of ice-cold RPMI 1640 were added to quench trypsinization. The samples were

transferred to 2.0 ml Eppendorf-Tubes and centrifuged (0.2 g, 5 min, 4 °C). The supernatant was discarded, and cells were washed twice with ice-cold DPBS under centrifugation. The cell pellets obtained after the last washing step were resuspended in 300 μ L ice-cold DPBS and kept on ice until measurement. Flow cytometry analyses were performed on a FACS Canto II (Becton Dickinson, Franklin Lakes, USA). The fluorescence of the CY5-labeled nanoparticles was excited at λ 633 nm and the emission was recorded using a λ 661/16 nm bandpass filter. The data was analyzed and plotted using Flowing software (v2.5.1, Cell Imaging and Cytometry Core, Turku Bioscience Centre, Turku, Finland, with the support of Biocenter Finland) and FCSalyzer software (open-source software by Sven Mostböck, Wien, Austria). Further evaluations were carried out based on the determined geometric mean of the fluorescence intensity.

Small Unilamellar Vesicle (SUV) Preparation

Lipid films (consisting of **A**: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC); **B**: POPC + 20 % 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS); **C**: POPC + 30 mol% cholesterol; **D**: POPC + 20 % POPS + 30 mol% cholesterol) were kindly provided by Prof. Claudia Steinem (Institute of Organic and Biomolecular Chemistry, University of Göttingen, Göttingen, Germany) and stored at 4 °C until use.^[62] For the preparation of small unilamellar vesicles, the films (0.4 mg) were allowed to swell in 600 μ L degassed DPBS for 25 min. Subsequently, the films were vortexed three times at 5 min intervals for 30 sec and then sonicated (Sonicator, cycle 4, power 60 %) for 30 min.^[63]

Preparation of Supported Lipid Bilayers (SLBs)

Silicon wafers (SiO₂: 5000 nm $\pm$ 5 %; polished/etched surface) (Active Business Company GmbH, Marquartstein, Germany) were rinsed twice with ethanol and Milli-Q water and then treated with a mixture of water, ammonia and hydrogen peroxide (5:1:1, v/v) at 70 °C for 20 min. After rinsing with Milli-Q water, the wafers were dried under the nitrogen flow and placed in the oxygen plasma (process parameters: pumping down: 01:30 min, gas supply: 05:30 min, plasma process: 00:30, venting: 01:30) (Plasma-Surface-Technology, Diener electronic, Ebhausen, Germany).^[63] For preparation of supported lipid bilayers, the SUV emulsions were allowed to spread over the hydrophilized silicon wafers.

Reflectometric Interference Spectroscopy (RIfS)

The spectra were recorded with a Flame-S-UV/Vis spectrometer (Ocean Optics, Dunedin, USA) at 2 sec intervals at RT as described in literature.^[64,65] In brief, a hydrophilized silicon wafer was inserted into a measuring cell and the system was flushed with DPBS for approximately 4 min to record a baseline. 300 μ L of the SUV emulsion were rinsed through the cell to spread as bilayer on the wafer. Subsequently, excess lipid phase was removed by flushing with DPBS. After the measurement signal

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stabilized, samples of COOH-NPs and 25 %-EXP-NPs were applied to the system in concentrations between 4.5 nM and 10 nM. Three different application regimes (a-c) were conducted:

a	Open circuit – flow measurements	Measurements under flow conditions in an open circuit: 2 ml of the sample were applied to the system, rinsed over the bilayer, and immediately discarded.
b	Closed circuit – flow measurements	Measurements under flow conditions in a closed circuit: The samples were circulated through the measuring cell for 1.5 h.
c	Measurements under static conditions	The measuring cell was flushed with sample and the pumps were then stopped to ensure that the bilayer in the measuring cell was incubated with the sample for 1 h.

At the end of each measurement, the system was flushed with DPBS and subsequently with Milli-Q water.

Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy (CLSM) images were recorded on an upright confocal laser microscope, LSM 710 Axio Examiner (Carl Zeiss MicroImaging GmbH, Jena, Germany), equipped with a 63x magnification water immersion objective.^[66] Fluorophores were excited at λ 488 nm (green) and λ 633 nm (red). The images were processed and analyzed using ZEN 3.5 blue edition software. In preparation for image acquisition, hydrophilized silicon wafers were glued under CLSM slides. Subsequently, the SUV emulsions (stained with 0.5 mol% ATTO 488) were applied to the wafers and allowed to spread for 1 h to form bilayers. The bilayers were then flushed six times with 1.0 ml DPBS. In the last rising step, 200 μ L DPBS were removed and displaced by 200 μ L of nanoparticle sample (COOH-NPs or 25 %-EXP-NPs) at a concentration of 10 nM. The bilayers were incubated with the samples for 30 min at 37 °C. Subsequently, the membranes were flushed at least six times with 1.0 ml DPBS. Images were taken at three different locations in the preparations and Z-stacks were recorded.

Software

The software used for the different experiments is listed below.

TopSpin 4.1.4 software was used for NMR processing and analysis (Bruker Corporation/Billerica/MA/USA, URL: <https://www.bruker.com/en/products-and-solutions/mr/nmr-software/topspin.html>).

Origin 2022b software (OriginLab Corporation/Northampton/Ma/ USA. URL: <https://www.originlab.com/2022b>) was used for data plotting and analyzing.

Flowing software (v2.5.1, Cell Imaging and Cytometry Core, Turku Bioscience Centre, Turku, Finland, with the support of Biocenter Finland. URL <https://flowingsoftware.com/download/>) and FCSalyzer software (open-source software by Sven Mostböck, Wien, Austria. URL <https://www.onworks.net/software/app-fcsalyzer>) were used for FACS data analyses and plotting.

R software (R Core Team (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>) was used for data plotting and statistical analysis.

Zen 3.5 blue edition software (Carl Zeiss Microscopy/Oberkochen/Germany) was used for confocal laser scanning microscopy image processing.

CorelDRAW 2023 Graphics Suite (Corel Corporation/Ottawa/Canada. URL <https://www.coreldraw.com/en/product/coreldraw/>) was used for graphical illustrations.

Python (Python Software Foundation. Python Language Reference, version 3.12, available at <https://www.python.org/downloads/release/python-3120/>) was used for calculations.

Blender (Blender Development Foundation (2002). Blender – a 3D modelling and rendering package, Blender version 4.2, available at <https://www.blender.org/>) was used for graphical illustrations.

Statistics

The statistical significance between means was evaluated with Welch's two sample t-test or one-way ANOVA with subsequent Bonferroni corrected post hoc paired t-test using R software. The data was tested for variance homogeneity using Levene test, before.

The data obtained from flow cytometry experiments were subjected to a Welch-ANOVA with subsequent Bonferroni corrected post hoc paired t-test, as the Levene test indicated variance heterogeneity ($\text{Pr}(> F) = 2.2 \cdot 10^{-16}$). Statistic testing was performed using R. The descriptive statistics, the Welch-ANOVA test statistics are shown below:

Descriptive statistics:

Group	Mean	SD	Median
100 %-EXP	5869.63	4345.47	4854
50 %-EXP	6074.87	5127.55	4770.74
COOH-	4824.1	3521.72	3898.72
MeO-	4907.24	4167.97	3763.02
Mixed charged-	3287.98	2644.2	2781
NH₂-	3352.3	3948.24	2776.8
ZW3-	3948.53	2629.49	3428.4

Welch-ANOVA ($\alpha = 0.05$): p-value < $2.2 \cdot 10^{-16}$

Bonferroni corrected post hoc paired t-test:

	<i>100 %-EXP</i>	<i>50 %-EXP</i>	<i>COOH-</i>	<i>MeO-</i>	<i>Mixed charged-</i>	<i>NH₂-</i>
<i>50 %-EXP</i>	$7.2 \cdot 10^{-11}$	-	-	-	-	-
<i>COOH-</i>	$< 2 \cdot 10^{-16}$	$< 2 \cdot 10^{-16}$	-	-	-	-
<i>MeO-</i>	$< 2 \cdot 10^{-16}$	$< 2 \cdot 10^{-16}$	0.16	-	-	-
<i>Mixed charged-</i>	$< 2 \cdot 10^{-16}$	$< 2 \cdot 10^{-16}$	$< 2 \cdot 10^{-16}$	$< 2 \cdot 10^{-16}$	-	-
<i>NH₂-</i>	$< 2 \cdot 10^{-16}$	$< 2 \cdot 10^{-16}$	$< 2 \cdot 10^{-16}$	$< 2 \cdot 10^{-16}$	0.52	-
<i>ZW3-</i>	$< 2 \cdot 10^{-16}$	$< 2 \cdot 10^{-16}$	$< 2 \cdot 10^{-16}$	$< 2 \cdot 10^{-16}$	$< 2 \cdot 10^{-16}$	$< 2 \cdot 10^{-16}$

The effect size was evaluated according to Cohen.^[39]

<i>Group 1</i>	<i>Group 2</i>	<i>Effect size</i>	<i>Magnitude</i>
100 %-EXP	50 %-EXP	-0.04318492	Negligible
100 %-EXP	COOH-	0.26434832	Small
100 %-EXP	MeO-	0.22603679	Small
100 %-EXP	Mixed charged-	0.71775015	Moderate
100 %-EXP	NH ₂ -	0.60634921	Moderate
100 %-EXP	ZW3-	0.53490665	Moderate
50 %-EXP	COOH-	0.28435938	Small
50 %-EXP	MeO-	0.24989546	Small
50 %-EXP	Mixed charged-	0.68315704	Moderate
50 %-EXP	NH ₂ -	0.59496069	Moderate
50 %-EXP	ZW3-	0.52184347	Moderate
COOH-	MeO-	-0.02154743	Negligible
COOH-	Mixed charged-	0.49329388	Small
COOH-	NH ₂ -	0.39341807	Small
COOH-	ZW3-	0.28173667	Small
MeO-	Mixed charged-	0.46393918	Small
MeO-	NH ₂ -	0.38302846	Small
MeO-	ZW3-	0.27512200	Small
<i>Mixed charged-</i>	<i>NH₂-</i>	<i>-0.01914467</i>	<i>Negligible</i>
Mixed charged-	ZW3-	-0.25050719	Small
NH ₂ -	ZW3-	-0.17774842	Negligible

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Supporting Information

The experiments on the supported lipid bilayers were conducted with the support of T. Heißenberg and J. Gerber-Nolte, under the supervision and expert advice of Dr. I. Mey and Prof. C. Steinem (Institute of Organic and Biomolecular Chemistry, Research Group, Prof. Steinem, Georg-August-University Göttingen).

Zwitterionic Head Group Synthesis and Characterization

PLA_{10K}-PEG_{5K} block copolymers with a zwitterionic sulfobetaine head group, PLA_{10K}-PEG_{5K}-sulfobetaine, were synthesized. The zwitterionic head group was built up on the deprotected amino-terminated polymer in a two-step synthesis. In the first step, PLA_{10K}-PEG_{5K}-4-(dimethylamino)butyric amid (referred to as PLA_{10K}-PEG_{5K}-ZW2) was synthesized by coupling 4-(dimethylamino)butyric acid hydrochloride to the amino-terminated polymer via EDC/NHS coupling chemistry. Therefore, 4-(dimethylamino)butyric acid hydrochloride (5 equiv.), EDC (10 equiv.) and NHS (5 equiv.) were dissolved in dry DMF and stirred for 1.5 h (500 rpm) at RT. Afterwards, a solution of the block copolymer, NH₂-PEG_{5K}-PLA_{10K} (1 equiv.), and DIPEA (17.5 equiv.) in DMF was added dropwise. The reaction mixture was stirred for 20 h at RT. For purification, the polymer was precipitated in diethyl ether and dried under the nitrogen flow. The resulting intermediate product (PLA_{10K}-PEG_{5K}-ZW2) was taken up in acetonitrile and dropped into Milli-Q water to form micelles for further purification by dialysis (using SpectraPor 6-8 kDa MWCO, RC membrane, Spectrum Laboratories, Inc, Los Angeles County, USA) against Milli-Q water for 24 h.

In the second synthesis step, the zwitterionic head group was synthesized by reacting the PLA_{10K}-PEG_{5K}-ZW2 polymer with 1,3-propanesultone as previously described in literature.^[1] Briefly, PLA_{10K}-PEG_{5K}-ZW2 was dissolved with the 9-fold excess of 1,3-propanesultone in THF and the reaction mixture was stirred (1500 rpm) for 48 h at RT. The resulting product was precipitated in diethyl ether and dried under nitrogen flow. For further purification, micelles were produced and dialyzed against Milli-Q water as described before. The synthesis product, hereafter referred to as PLA_{10K}-PEG_{5K}-sulfobetaine (PLA_{10K}-PEG_{5K}-ZW3), was lyophilized using single-chamber, bench-top freeze dryer Alpha 2-4 LSC plus (Christ, Osterode am Harz, Germany) and subsequently characterized.

Verification of the Chemical Conversion

The conversion of the free amino group of the reactant PLA_{10K}-PEG_{5K}-NH₂ (first synthesis step) was proven via Fluram assay. Therefore, PLA_{10K}-PEG_{5K}-NH₂ was used as positive control. PLA_{10K}-PEG_{5K}-COOH and 4-(dimethylamino)butyric acid hydrochloride were used as negative control (Figure S1). Furthermore, PLA_{10K}-PEG_{5K}-NH₂, PLA_{10K}-PEG_{5K}-ZW2 and PLA_{10K}-PEG_{5K}-ZW3 were analyzed by ¹H-NMR and FTIR. ¹H-NMR spectra were recorded on a Bruker Avance III HD 400 operating at 400 MHz. The polymers were dissolved in deuterated chloroform (CDCl₃). FTIR spectra were recorded on an Agilent Technologies Cary 630 FTIR (Agilent, Santa Clara, USA), all samples were measured twice. The evaluation of the ¹H-NMR and IR data did not provide clear result regarding the success of the synthesis (*data not shown*). This was probably attributed to the low molecular weight of the sulfobetaine head group (253.10 g mol⁻¹) compared to molecular weight of the associated polymer chain (15,000 g mol⁻¹).

For enhanced characterization and verification of the synthesis conditions, the synthesis was repeated on NH₂-PEG_{5K} chains (Jenkem Technology, USA, Mn 5000 ± 500 g mol⁻¹, stated on

certificate of analysis). The resulting polymers (reactant, intermediate and product) were characterized by FTIR-spectra and via Fluram assay. (Figure S2).

For further characterization, the sulfobetaine head group was synthesized isolated. For solubility reasons, this synthesis was performed in Milli-Q water. Therefore, 4-(dimethylamino)butyric acid hydrochloride (1 equiv.) was dissolved together with 1,3-propanesultone (2 equiv.) in MilliQ-water. The reaction mixture was stirred for 24 h at RT. The resulting product was characterized by MS analysis. Mass spectrometry analysis was performed on a Q-TOF 6540 ultrahigh definition (Agilent Technologies, Waldbronn, Germany) using an electrospray ionization source (ESI) (Figure S3).

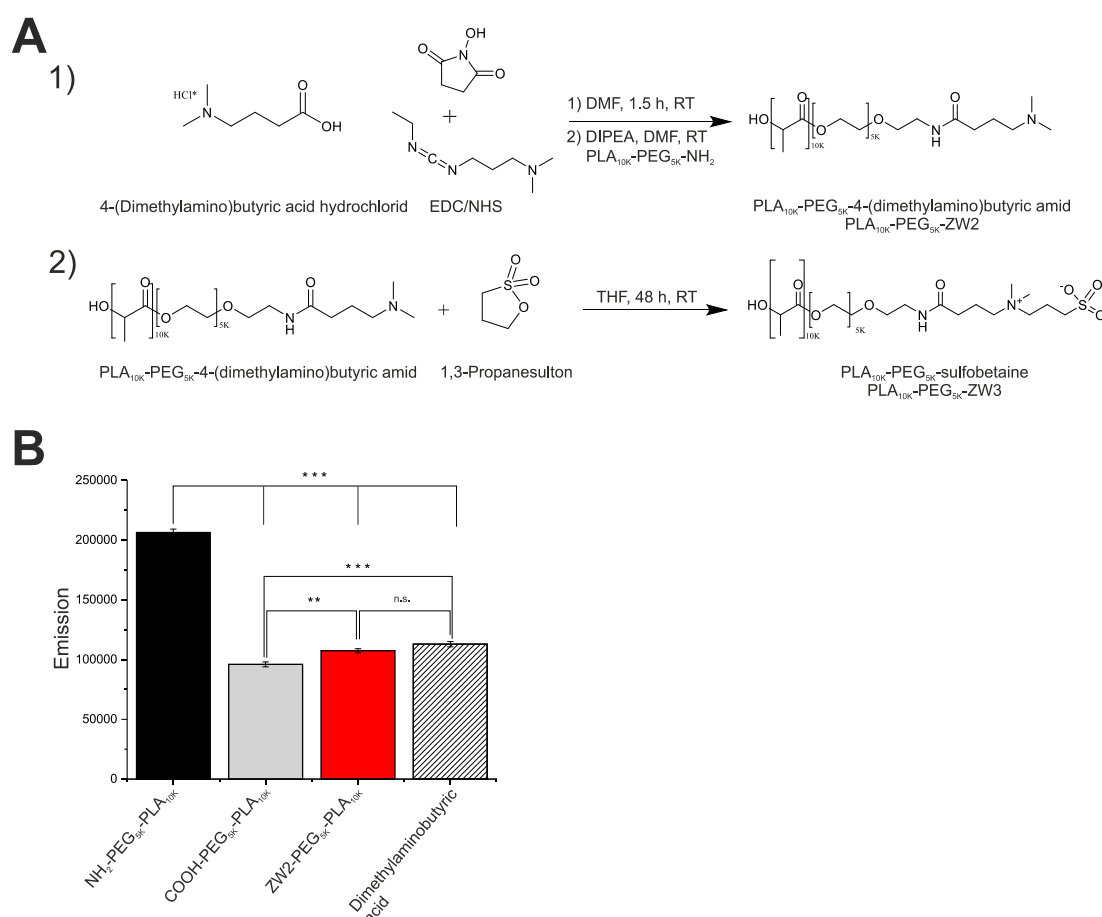


Figure S 1 Synthesis and characterization of PLA_{10K}-PEG_{5K}-ZW2. (A) Reaction equation of the two-step synthesis of the zwitterionic head group (ZW3-) on PLA_{10K}-PEG_{5K}-NH₂. (B) Verification of the chemical conversion of the free amino group of PLA_{10K}-PEG_{5K}-NH₂ via Fluram assay. PLA_{10K}-PEG_{5K}-NH₂ was used as positive control. PLA_{10K}-PEG_{5K}-COOH and 4-(dimethylamino)butyric acid hydrochloride were used as negative controls. Polymer micelles of PLA_{10K}-PEG_{5K}-NH₂, PLA_{10K}-PEG_{5K}-COOH and PLA_{10K}-PEG_{5K}-ZW2 were prepared by nanoprecipitation method using equal polymer concentrations. The emission detected for PLA_{10K}-PEG_{5K}-ZW2 differed significantly from the emission of the educt NH₂-PEG_{5K}-PLA_{10K} (p-value = $4.4 \cdot 10^{-10}$) and was at the level of dimethylaminobutyric acid. The derived emission values were compared via one-way ANOVA with subsequent Bonferroni corrected post hoc paired t-test after testing for variance homogeneity using Levene test. Results represented as mean $\pm$ SD (n = 3, levels of statistical significance are indicated as n.s. – not significant, *p $\leq$ 0.05, **p $\leq$ 0.01, ***p $\leq$ 0.001).

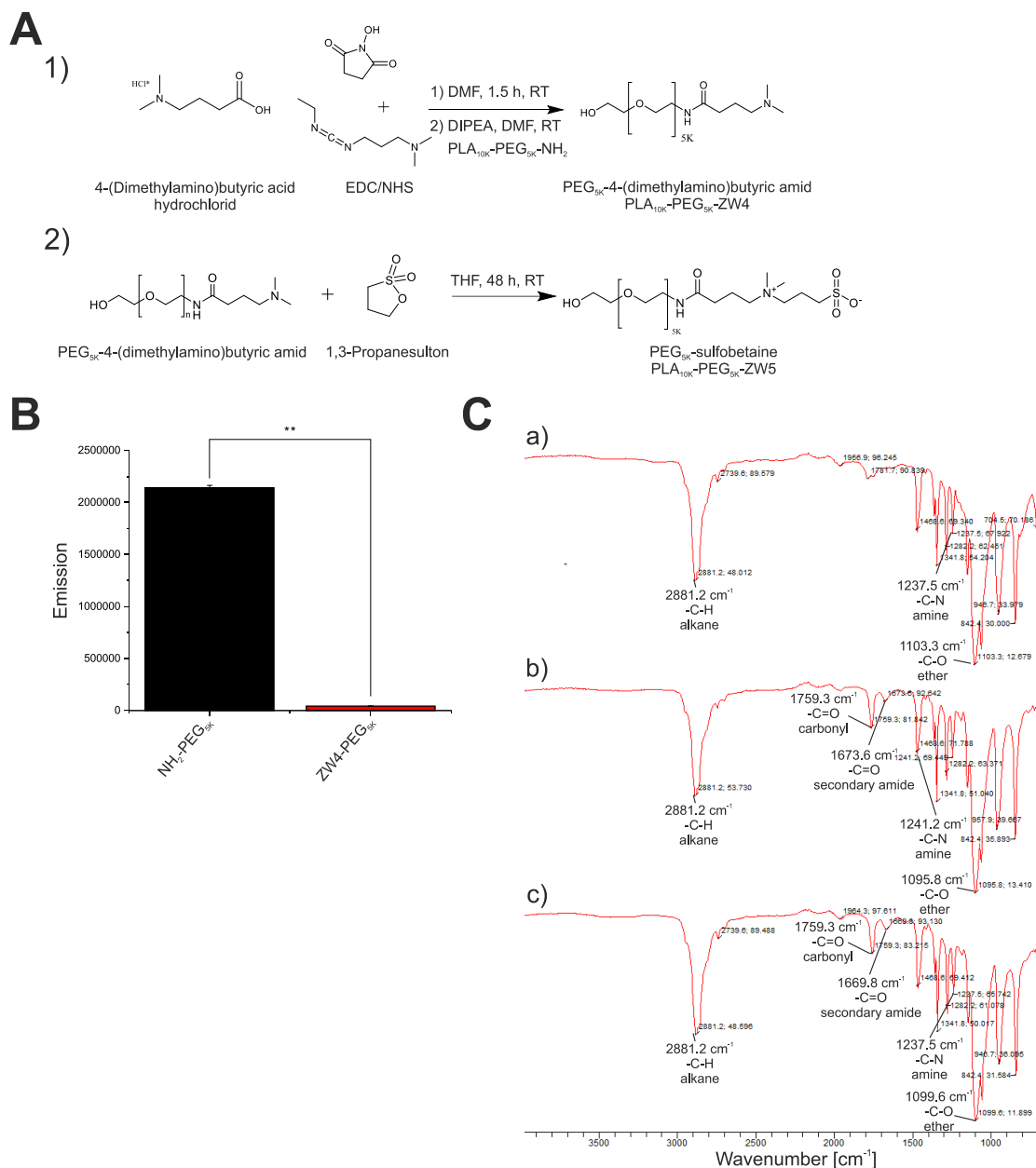


Figure S 2 Synthesis and characterization of PEG_{5K}-ZW4 and PEG_{5K}-ZW5. (A) Reaction equation for the two-step synthesis of the zwitterionic head group (sulfobetaine) on NH₂-PEG_{5K} polymer. (B) Verification of the chemical conversion of the free amino group of the PEG_{5K}-NH₂ via Fluram assay. The data was compared using Welch's t-test (p-value = 0.0073). The results indicate a statistically significant reduction of the emission from the PEG_{5K}-ZW4 compared to NH₂-PEG_{5K}. Results presented as mean $\pm$ SD (n = 3, levels of statistical significance are indicated as n.s. – not significant, *p $\leq$ 0.05, **p $\leq$ 0.01, ***p $\leq$ 0.001). (C) FTIR spectra of a) NH₂-PEG_{5K}, b) ZW4-PEG_{5K}, and c) ZW5-PEG_{5K}. Peaks for carbonyl groups (1759.3 [cm⁻¹] v (-C=O)) and secondary amide (1673.6 [cm⁻¹] / 1669.8 [cm⁻¹] v (-C=O)) were found in the spectra of PEG_{5K}-ZW4 and PEG_{5K}-ZW5. However, no peak in the spectrum of PEG_{5K}-ZW5 could be clearly assigned to the sulfoxide group (1070-1030 [cm⁻¹] v (-S=O)). The peak may be overlapping with the ether peak of the PEG_{5K} chain (1300-1020 [cm⁻¹] v (-C-O-C)).

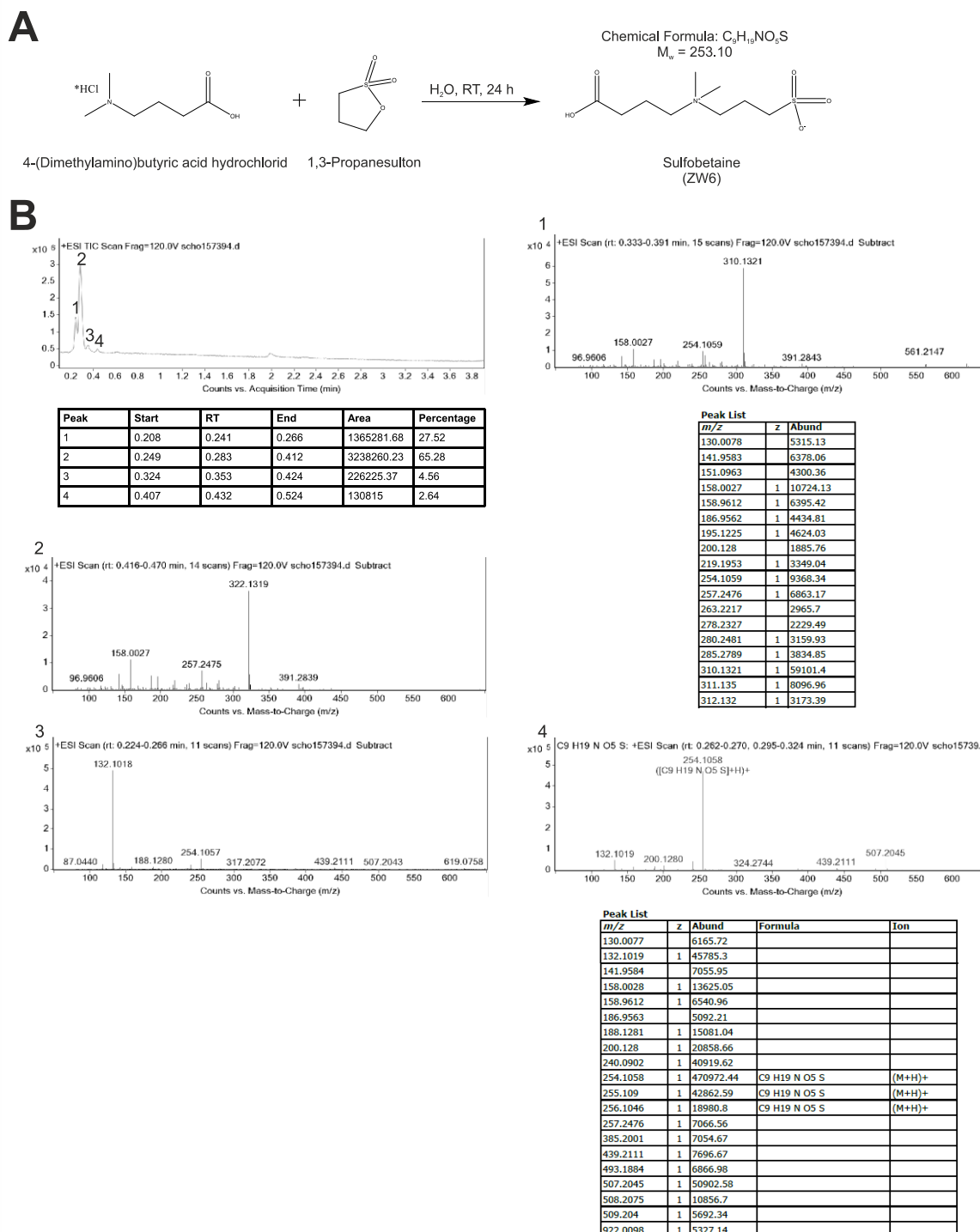


Figure S 3 Synthesis and characterization of the isolated sulfobetaine (ZW6). (A) Reaction equation for the sulfobetaine (ZW6) synthesis performed in Milli-Q water. (B) LC-MS chromatogram showing peaks of the reactant (peak 2 and 3) and the product (peak 1 and 4). The mass of 254.1058 (MH^+) corresponding to the product and the mass of 310.1321 (MH^+) corresponding to the product associated with counterions (NaCl) were found. Furthermore, the mass of 322.1319 (MH^+) corresponding to the reactant, 4-(dimethylamino)butyric acid hydrochloride, and the mass of 132.1018 (MH^+) corresponding to the reactant dissociated from its counterions were found.

Table S1

	<i>DLS</i>				<i>NTA</i>		
	Size, d_{NP} [nm]	SD Size [nm]	PDI	Zetapotential [mV]	Size, d_{NP} [nm]	SD Size [nm]	Conc. [NPs/ ml]
ZW3-	88.52	3.866	0.139	7.59	93.1 ± 1.9	35.7 ± 7.6	$4.12 \cdot 10^{12} \pm 4.39 \cdot 10^{11}$
NH ₂ -	93.04	0.509	0.171	2.08	78.2 ± 2.7	27.9 ± 4.5	$1.14 \cdot 10^{13} \pm 5.10 \cdot 10^{11}$
Mixed charged-	100.1	0.456	0.131	-6.67	90.1 ± 1.5	35.1 ± 1.1	$1.31 \cdot 10^{13} \pm 1.58 \cdot 10^{12}$
MeO-	80.03	1.297	0.162	0.056	104.3 ± 4.4	56.0 ± 6.2	$2.78 \cdot 10^{12} \pm 3.19 \cdot 10^{11}$
COOH-	105.8	0.854	0.129	-15.48	93.0 ± 0.2	30.9 ± 0.5	$4.38 \cdot 10^{12} \pm 3.91 \cdot 10^{11}$
50 %-EXP/ COOH-	105.5	0.404	0.139	-7.48	100.4 ± 0.7	29.5 ± 4.8	$2.46 \cdot 10^{12} \pm 8.08 \cdot 10^{10}$
100 %-EXP	90.31	0.872	0.196	-4.83	78.8 ± 2.1	28.5 ± 2.2	$5.19 \cdot 10^{12} \pm 5.57 \cdot 10^{11}$

Table S2

<i>Saturation binding model</i>	
<i>Spacer chain head group</i>	<i>calc. K_i [nM]</i>
ZW3-	0.09895 ± 0.04378
NH ₂ -	0.12327 ± 0.14068
Mixed charged-	0.14604 ± 0.08265
MeO-	0.04913 ± 0.0278
COOH-	0.06788 ± 0.02936
50 %-EXP/ COOH-	0.06897 ± 0.02202
100 %-EXP	0.09572 ± 0.02411

Table S 1 Nanoparticle data on size (dh), polydispersity index (PDI), zeta potential, and concentration obtained from dynamic light scattering (DLS) and single nanoparticle tracking analysis (NTA). The nanoparticles were manufactured with CY-5 labelled PLGA used as core material. Data were obtained from at least three replicate runs. **Table S 2 K_i values of the nanoparticles derived from a classical saturation binding model.** As the binding data was not corrected by for non-specific binding, the obtained K_i values reflect specific and non-specific binding of the nanoparticles toward the target cells.

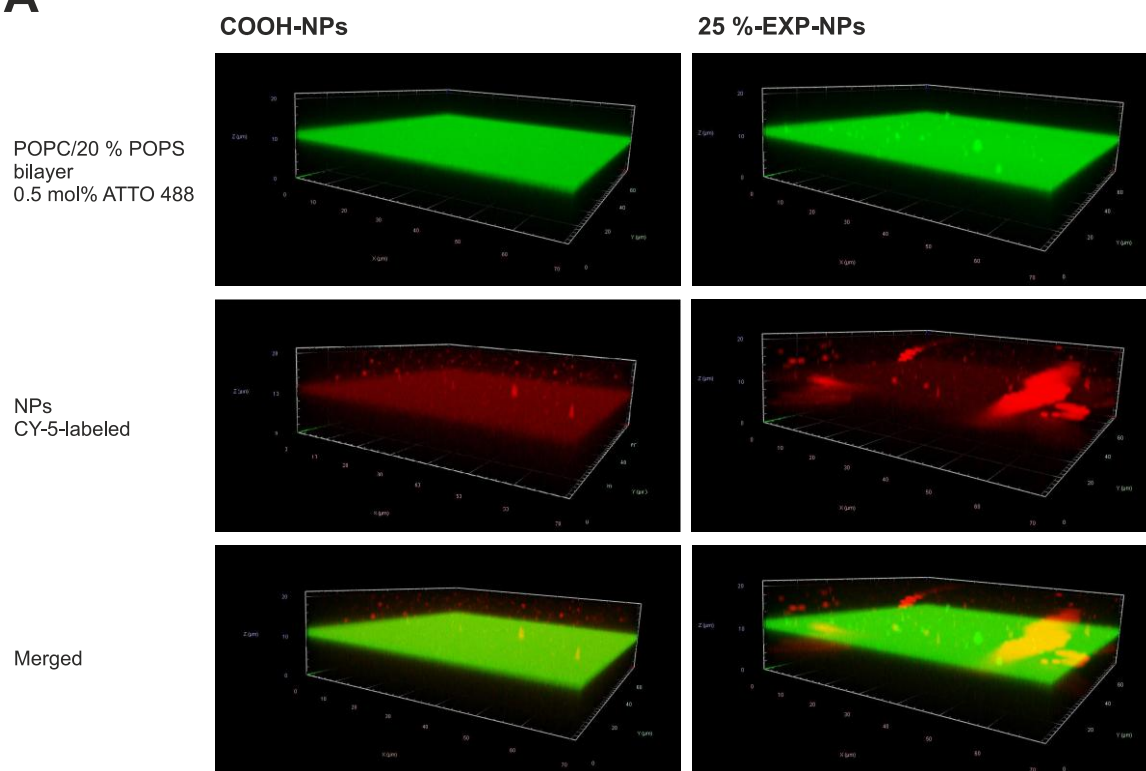
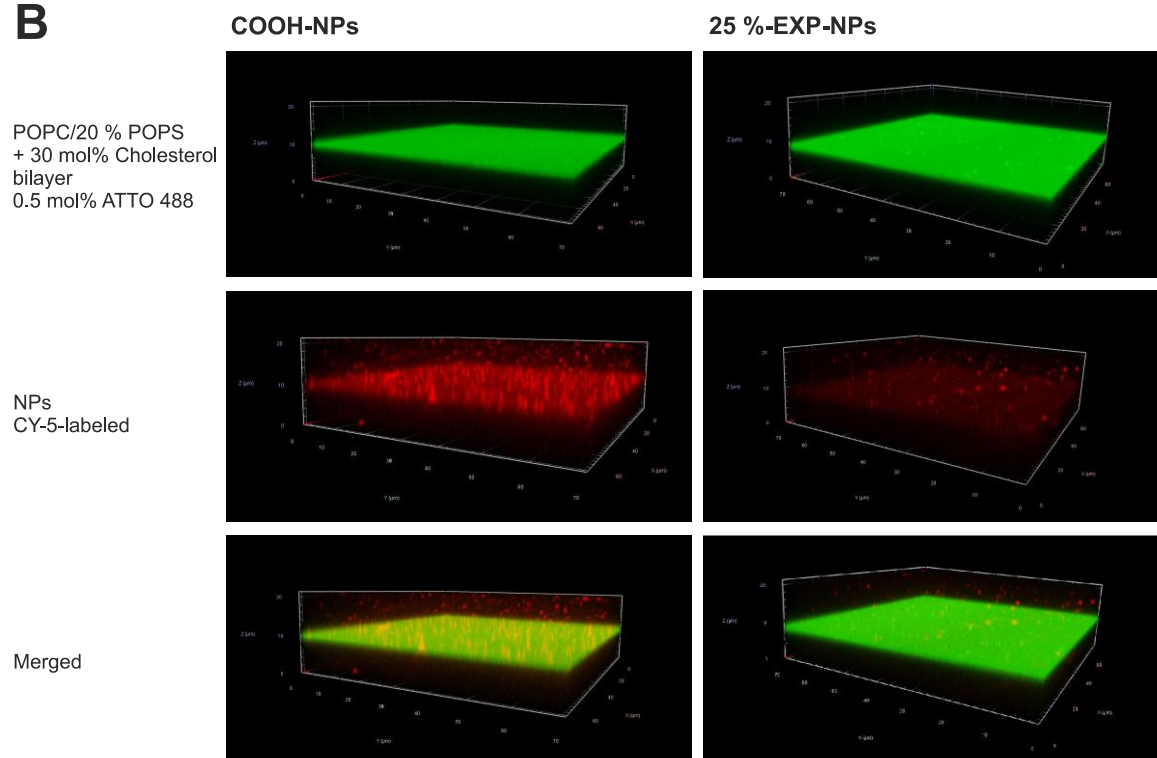
A**B**

Figure S 4 Localization of 25 %-EXP-NPs and COOH-NPs relative to the supported lipid bilayers. The lipid bilayers were stained with 0.5 mol% ATTO 488 (green) and the NPs were labeled with CY-5 (red). (A) 3D images on POPC/20 % POPS bilayers. (B) 3D images on POPC/20 % POPS + 30 mol% cholesterol bilayers. Due to the acquired contrast and color settings, only images taken on the same bilayer type were comparable.

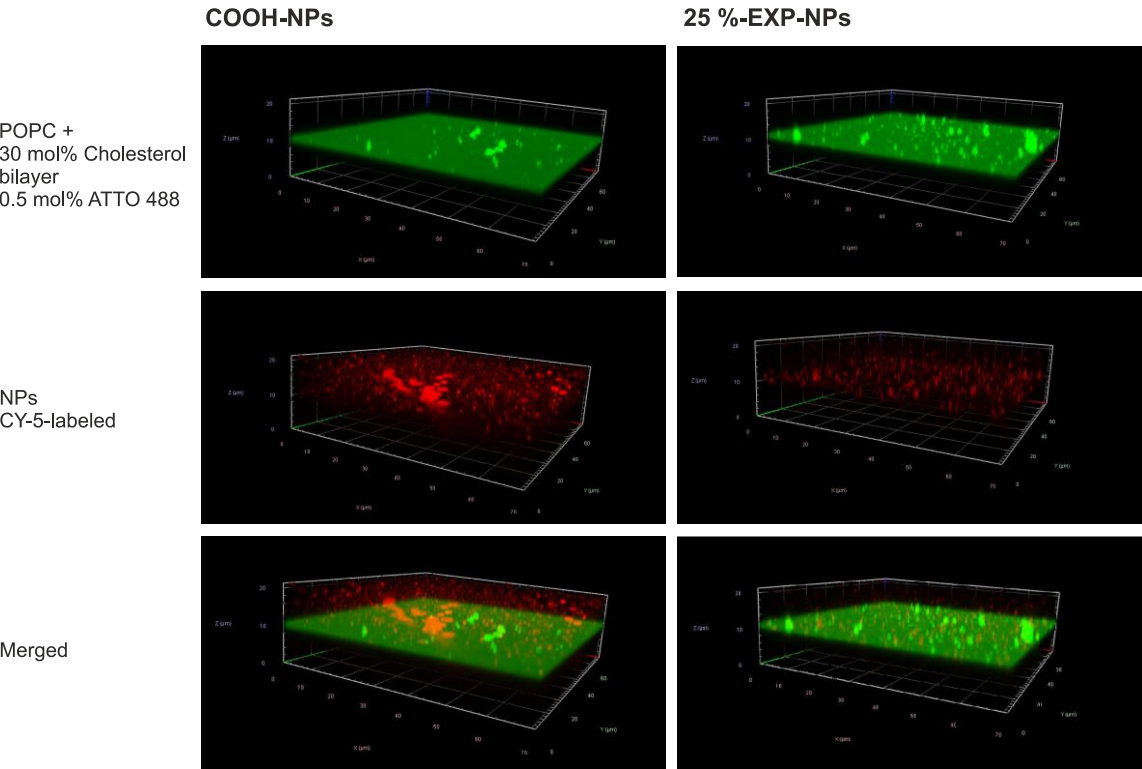


Figure S 5 Localization of 25 %-EXP-NPs and COOH-NPs relative to the supported lipid bilayers composed of POPC + 30 mol% cholesterol bilayers. The lipid bilayers were stained with 0.5 mol% ATTO 488 (green) and the NPs were labeled with CY-5 (red).

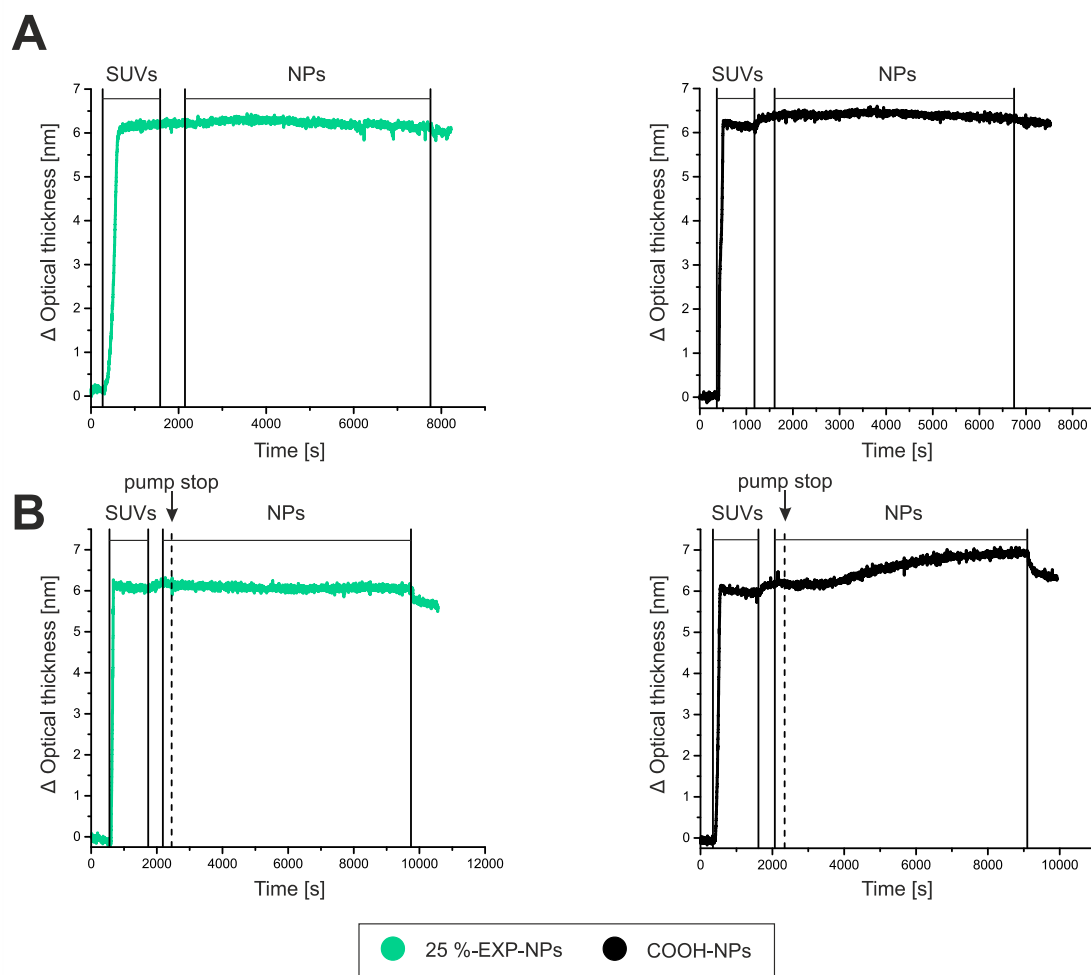


Figure S 6 Reflectometric interference spectroscopy experiments on POPC membranes under (A) closed circuit - flow measurements conditions (B) static conditions.

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Chapter 5

Rethinking Thin-Layer Chromatography for Screening Technetium-99m Radiolabeled Polymer Nanoparticles

Manuscript

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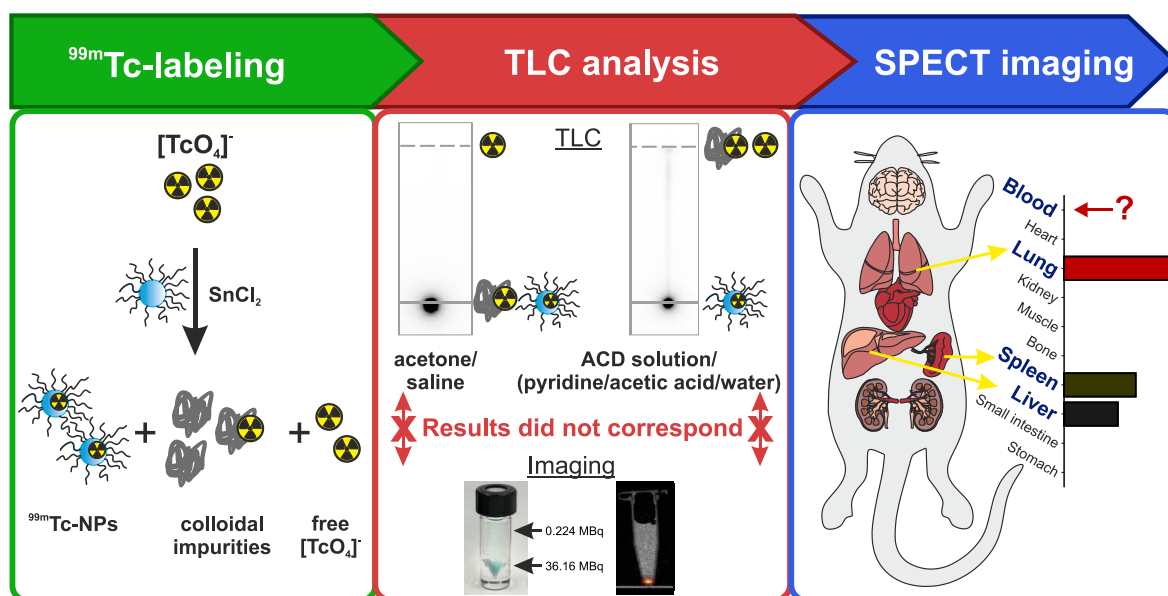
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K. S. and X. C. contributed equally to this work (shared first authors). K. S. and X. C. designed and performed the research, analyzed the data and wrote the manuscript. K. S. performed the experiments related to nanoparticle preparation and characterization and conducted TLC experiments with nanoparticles and polymers. X. C., T. S. and A.-P. A.-L. conducted the radiolabeling experiments and animal studies. J. L. synthesized the PLGA-CY-5. T. H. and A. G. supervised the project, provided scientific input during the complete process, supported the manuscript preparation and reviewed the final manuscript.

Abstract

Thin-layer chromatography (TLC) is commonly employed to screen technetium-99m labeled polymer nanoparticle batches for unreduced pertechnetate and radio-colloidal impurities. Although this method is widely accepted, our findings applying radiolabeled PLGA/PLA-PEG nanoparticles underscore its lacking transferability between different settings and its limitations as a standalone quality control tool. While TLC results may appear similar for purified and radio-colloid containing nanoparticle formulations, the batches' in vivo behavior can vary significantly, demonstrated by discrepancies between TLC results and single-photon emission computed tomography (SPECT) and biodistribution data. This highlights the urgent need for a case-by-case evaluation of TLC methods for each specific nanoparticle type. Our study revealed that polymer nanoparticles cannot be considered analytically uniform entities in the context of TLC analysis, emphasizing the complex interplay between nanoparticle composition, radiolabeling conditions, and subsequent biological behavior.



Graphical Abstract

Introduction

Nanoparticles (NPs) have emerged as a promising platform technology for drug delivery^[1,2] and diagnostics,^[3] but their unique properties pose challenges for traditional characterization methods like liquid chromatography-mass spectrometry. To overcome these limitations, radioactive labeling is often employed for in vivo studies, enabling real-time tracking with high sensitivity.^[4,5] Technetium-99m (^{99m}Tc) is a popular radionuclide due to its short half-life, ideal photon energy for single-photon emission computed tomography (SPECT) imaging, and availability from ⁹⁹Mo/^{99m}Tc generators.^[4] For certain polymer NPs, direct incorporation of radionuclides into the NP structure is feasible without additional chelating groups.^[6–8] However, this requires reducing pertechnetate (^{99m}TcO₄[−]) to a lower oxidation state.^[9] While tin chloride (SnCl₂) is commonly used as a reducing agent^[7,8,10–12] due to its mild, nanoparticle-friendly reaction conditions,^[6,9] the radiolabeling process must be carefully optimized for each NP type. This optimization is crucial as various radiochemical impurities can occur during the labeling process, including unreduced ^{99m}TcO₄[−] and different colloidal species such as ^{99m}Tc(IV)-oxide-colloids and ^{99m}Tc(IV)-Sn-colloids.^[9,13] Moreover, the formation of tin oxide and hydroxide colloids are suspected to cause attachment of the radiolabeled polymeric NPs and can further complicate the system.^[14] These colloidal impurities, collectively referred to as hydrolyzed-reduced technetium-99m (HR-^{99m}Tc), can significantly impact radiolabeling efficiency and biodistribution data if undetected.^[4]

To address this challenge, a comprehensive analysis of impurities in ^{99m}Tc-NP batches is essential. Radio-thin layer chromatography (TLC) is a widely adopted method for assessing radiochemical purity of radiopharmaceuticals due to its simplicity and speed.^[15] However, the application of TLC analysis to NPs is not standard practice in other fields.^[16] To distinguish ^{99m}Tc-NPs from ^{99m}TcO₄[−] and HR-^{99m}Tc, two sets of developing systems for TLC analysis are commonly employed.^[12,13,17,18] An organic solvent (e.g. acetone or methyl ethyl ketone) and saline are used to identify ^{99m}TcO₄[−]. Subsequently, acidic developing solvents are utilized to separate ^{99m}Tc-NPs from HR-^{99m}Tc. Popular choices for these acidic solvents include anticoagulant citrate dextrose (ACD) solution (pH 5)^[17] and a pyridine, acetic acid and water mixture (Py/AA/H₂O). While ACD solution has been reported to develop HR-^{99m}Tc, the Py/AA/H₂O mixture is employed to develop ^{99m}Tc-NPs (Figure 1A). By comparing the results from both TLC plates, researchers can estimate the relative proportions of different species presented in the reaction mixture.^[17]

The current study aims to examine and scrutinize the general applicability and transferability of the TLC analytical method outlined above. We selected polymer poly(D,L-lactide-co-glycolide) (PLGA)/poly(ethylene glycol)-*b*-poly(D,L-lactide) (PLA-PEG) core-shell NPs for radiolabeling using ^{99m}Tc and SnCl₂, as they serve as a valuable model due to their composition of polymers that are widely used for NPs production.^[18,19]

Results and Discussion

When establishing a ^{99m}Tc radiolabeling protocol for cyanine-5-amine (CY-5)-tagged core-shell NPs using SnCl_2 as reducing agent, it was discovered that the formation of $\text{HR-}^{99m}\text{Tc}$ (Figure S1) was not reflected in the TLC analysis results (Figure S2), as per the adopted method from the literature (Figure 1A). Based on these observations, control experiments were systematically conducted to investigate the transferability of the TLC analysis.

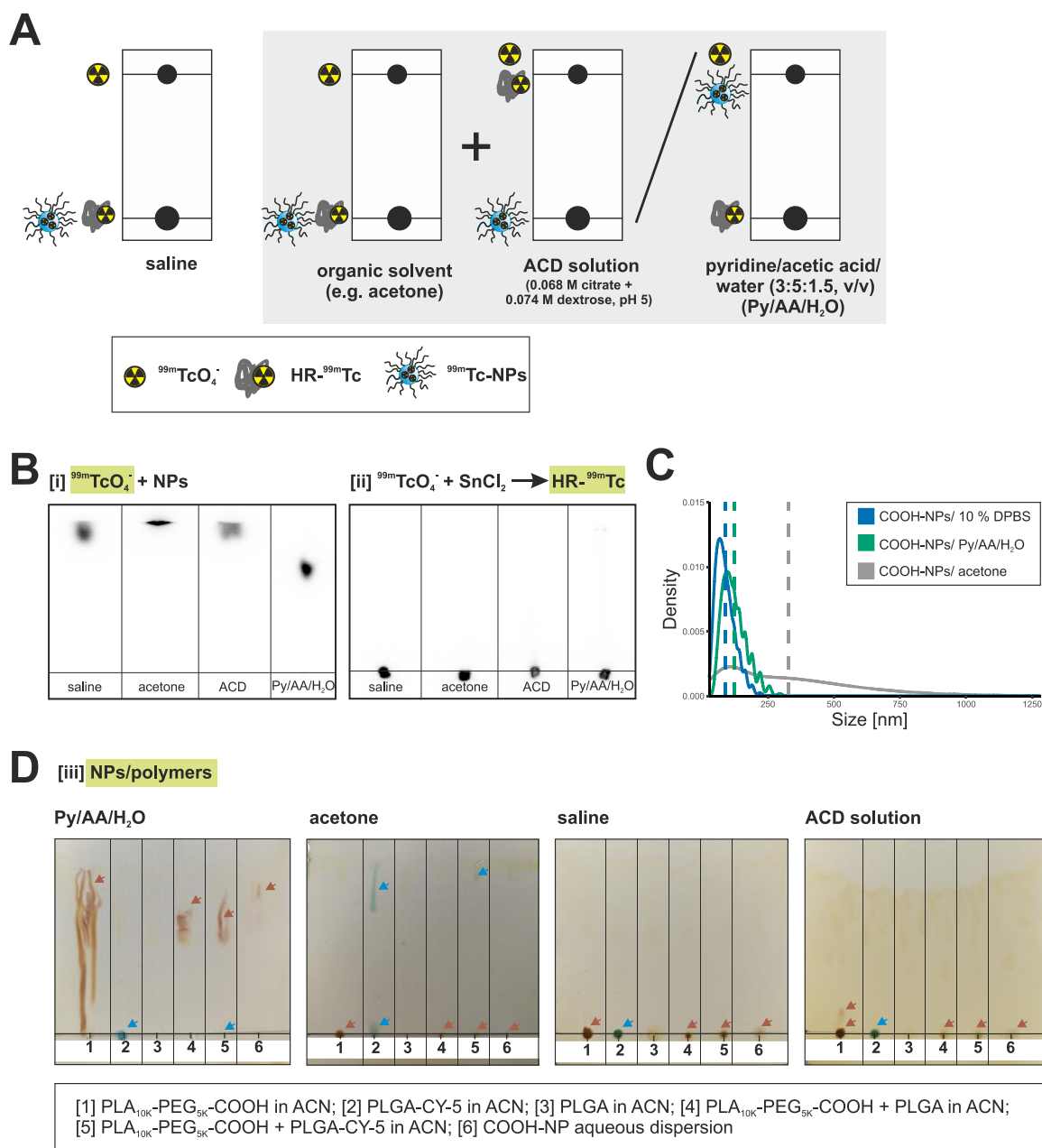


Figure 1 Thin-layer chromatographic methods and control experiments. (A) Illustration of the thin-layer chromatographic methods described in the literature^[11,12,17,20] for the analytical separation of $^{99m}\text{TcO}_4^-$, $^{99m}\text{Tc-NPs}$ and $\text{HR-}^{99m}\text{Tc}$. (B) TLCs of NPs + $^{99m}\text{TcO}_4^-$ in the absence of a reducing agent and TLCs of $^{99m}\text{TcO}_4^- + \text{SnCl}_2$ (solubilized in HCl) in the absence of NPs. (C) Density plots of NP size distributions. The NPs

were diluted with 10 % DPBS or Py/AA/H₂O or acetone. The mean values are highlighted as vertical dashed lines corresponding to the color of the density curves. (D) TLCs of polymers and NPs developed in various developing solvents, followed by staining with iodine vapor. Blue arrows indicate spots visible before staining, while brown arrows represent spots revealed after iodine staining. PLGA [3] serves as a negative control for staining with iodine vapor.

The developing of $^{99m}\text{TcO}_4^-$ ([i]), HR- ^{99m}Tc ([ii]) as well as NPs and their constituent polymers ([iii]) on the TLC plates using the solvents described in the literature was analyzed independently. Initially, NPs and $^{99m}\text{TcO}_4^-$ were investigated in the absence of SnCl_2 , confirming that unreacted $^{99m}\text{TcO}_4^-$ was developed with all the solvents described (Figure 1B). As HR- ^{99m}Tc is mainly formed in the absence of a suitable ligand or in sufficient quantities of the ligand,^[9] the development behavior of a reaction mixture of $^{99m}\text{TcO}_4^-$ and SnCl_2 without addition of NPs was analyzed (Figure 1B). The result showed that HR- ^{99m}Tc remained at the application point in all solvents, contradicting the previous reports.^{[17],[21]} In the literature it was suggested that HR- ^{99m}Tc could be solubilized and developed with ACD solution due to complexation with the citrate it contains. Further orthogonal experiments using SnCl_2 solubilized in citric acid instead of HCl confirmed the formation of a ^{99m}Tc -citrate complex that could be developed with saline, ACD and Py/AA/H₂O mixture but not with acetone (Figure S3). A homogeneous distribution of radioactivity in the reaction vessel was observed, as recorded by a SPECT phantom (Figure S3). These findings emphasize that the formation of ^{99m}Tc -citrate complex is favored over the formation of HR- ^{99m}Tc and ^{99m}Tc -NPs. Nevertheless, it cannot be assumed that such complex forms quantitatively from HR- ^{99m}Tc and develops with citrate-containing ACD solution. In addition, the development behavior of the NPs under consideration was analyzed. These NPs consist of a lipophilic PLGA core (Figure S4) associated with PLA-PEG copolymer (Figure S5). The core and shell are attached by the lipophilic nature of PLGA and PLA in the aqueous nanoparticle dispersion and the PEG chains form the shell (Figure S6, Figure S7). When applied to the TLC plates, polymer NPs can no longer be considered as a singular analytical unit but rather as the sum of the polymers that they are composed of. The presumed disintegration of the nanoparticle structure was proven by dynamic light scattering (DLS) measurements of NPs diluted with organic solvents. The mean nanoparticle size shifted from 89.1 ± 36.2 nm (in 10 % Dulbecco's phosphate-buffered saline (DPBS)) to 123.3 ± 45.8 nm and 329.6 ± 242.3 nm when diluted with Py/AA/H₂O mixture and acetone, respectively (Figure 1C, Figure S8A). Therefore, in addition to NPs, polymer mixtures and individual polymers were also applied to the TLC plates to investigate their retention behavior. The fluorescent CY-5-tag facilitated the visual identification of labeled PLGA, while the PLA-PEG block-co-polymer was visualized on the TLC plates through iodine staining. The results indicate that purely aqueous solvents (saline and ACD solution) cannot develop the polymers or the NPs ($R_f = 0$). Contrarily, mobile phases consisting of organic solvents lead to a partial development of the polymers ($R_f = 0.2\text{--}0.8$) (Figure 1D, Figure S8). PLA-PEG was not developed in acetone ($R_f = 0$), but in the Py/AA/H₂O mixture ($R_f = 0.6\text{--}0.8$). In contrast, PLGA was not developed in the Py/AA/H₂O mixture ($R_f = 0$), but partially in acetone ($R_f = 0.6\text{--}0.9$). One reason for the partial development in acetone (Figure S8) could be the migration and separation of PLGA due to different molecular weight (Resomer RG 502, M_w 7000-17000).^[22] This was supported by the recorded HPLC chromatograms

(Figure S4). Although the phase transition between the stationary and mobile phase is a driving force for the separation of the analytes during TLC development, these results cannot be explained simply by the solubility of the polymers in the mobile phases. An estimation of the solubility of both polymers in the solvents using the “Hansen parameters” showed a comparatively good solubility in the organic solvents (Figure S9). This underlines that the retention in multicomponent systems depends not only on the solubility in the mobile phase, but also on several other factors, such as association effects and the properties of the stationary phase.^[23] These results highlight the complexity of predicting TLC behavior of polymeric systems.

To assess the impact of these findings on radiolabeling and biodistribution, we compared three radiolabeling conditions: a standard reduction with SnCl_2 , an optimized reduction with SnCl_2 , and a reduction with $\text{Na}_2\text{S}_2\text{O}_4$ (Table 1). The first reaction condition with SnCl_2 at room temperature was expected to form $\text{HR-}^{99\text{m}}\text{Tc}$ (condition [1]). The second condition, still using SnCl_2 as reducing agent but at 37 °C for 30 min, was validated for good labeling efficiency (condition [2]). The third one, using $\text{Na}_2\text{S}_2\text{O}_4$ as reducing agent, could prevent the formation of $\text{HR-}^{99\text{m}}\text{Tc}$ (condition [3]).

	Reducing agent	Reaction conditions	Expected colloid formation	SPECT imaging	DLS measurements (dh)
[1]	SnCl_2	RT, 30 min	Yes	clotted radioactivity → colloids	colloids: 1.99-5.56 μm
[2]	SnCl_2	37°C, 30 min	No	homogeneously distributed radioactivity	colloids: -
[3]	$\text{Na}_2\text{S}_2\text{O}_4$	37°C, 30 min	No mixed colloids ($^{99\text{m}}\text{Tc-Sn(OH)}_2$)	homogeneously distributed radioactivity	colloids: -

Table 1 Overview of the experimental conditions and results.

Although all three labeling conditions showed the same results in the radio-TLCs, with no development of sample spots ($R_f = 0$) in any of the eluent systems described above (Figure 2B), their SPECT imaging demonstrated different characteristics (Figure 2A). In the sample from condition [1], the radioactivity was concentrated at the bottom of the reaction vessel. This observation contradicted the presence of not only free, unbound $^{99\text{m}}\text{Tc}$ and unreduced $^{99\text{m}}\text{TcO}_4^-$, but also radiolabeled NPs (89.1 $\pm$ 36.2 nm) according to Stokes' law. DLS measurements further confirmed the presence of colloidal radioactive species adjacent to the unlabeled NPs, detecting colloids in the size range of 2 to 6 μm (dh) (Figure 2C, Figure S10). In contrast, samples from conditions [2] and [3] exhibited a homogeneous distribution of radioactivity in the reaction vessels (Figure 2A). Thus, in the current setting, the TLC analysis could not contribute to the differentiation of the samples or the characterization of the present radioactive species.

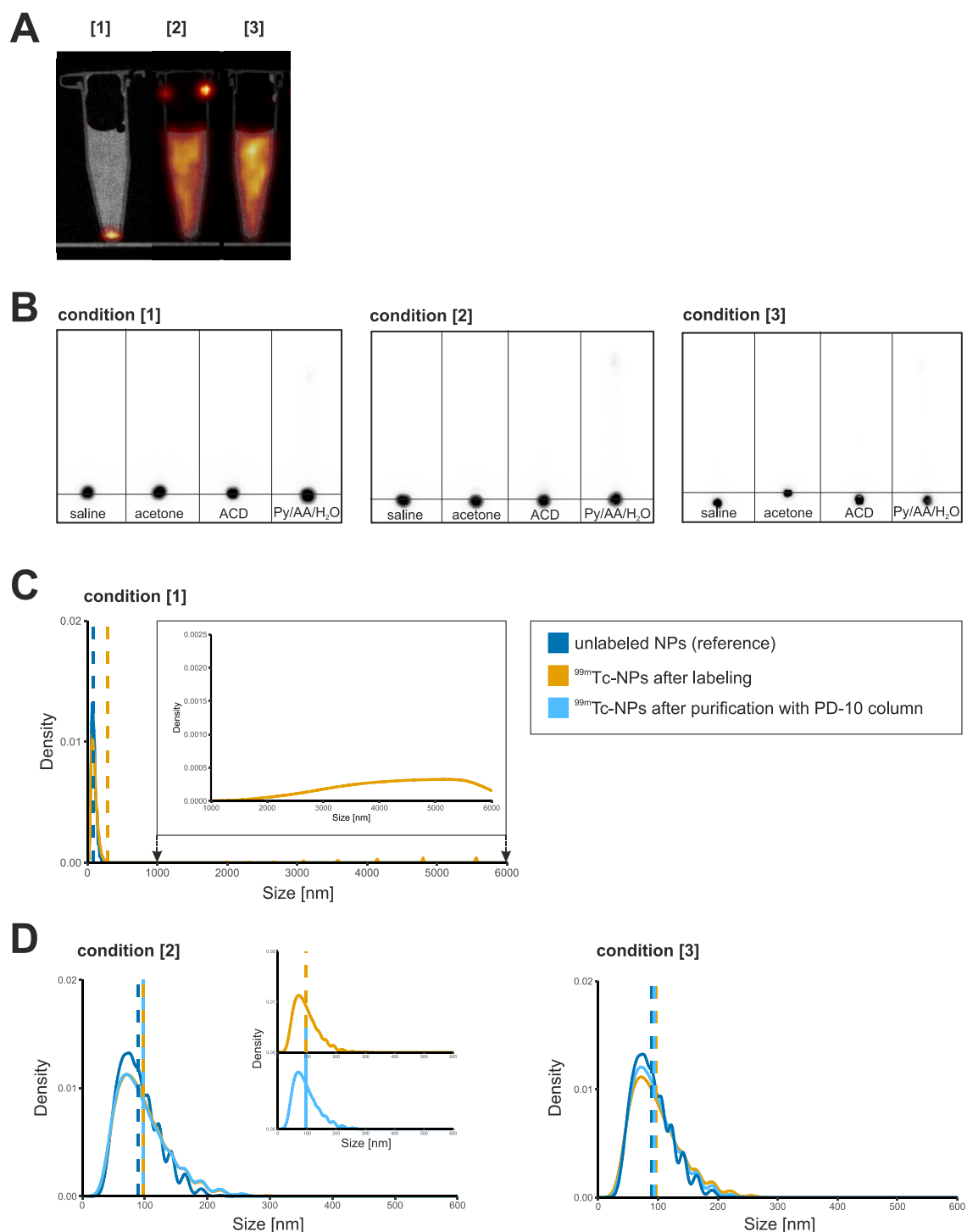


Figure 2 TLCs and SPECT/CT fusion imaging of NPs radiolabeled under different conditions along with the corresponding density plots of NP size distributions. (A) SPECT imaging fused with CT of the reaction vessels from experimental conditions [1]-[3]. (B) TLCs of the reaction mixtures from labeling condition [1]-[3]. (C) DLS measurements corresponding to condition [1]. DLS data of unlabeled NPs was plotted as reference. The mean values were highlighted as vertical dashed lines corresponding to the color of the density curves. (D) DLS measurements corresponding to experimental condition [2]-[3], before (yellow) and after (light blue) purification with PD-10 column. (In the case of condition [2], the density curves were additionally displayed separately for better visibility.)

Chapter 5

To confirm that the ^{99m}Tc in samples from conditions [2] and [3] was indeed bound to the NPs and not present as free species, we subjected the samples to size-exclusion chromatography using PD-10 column. The first five fractions (1 mL each) were collected and analyzed for radioactivity and NP concentration.

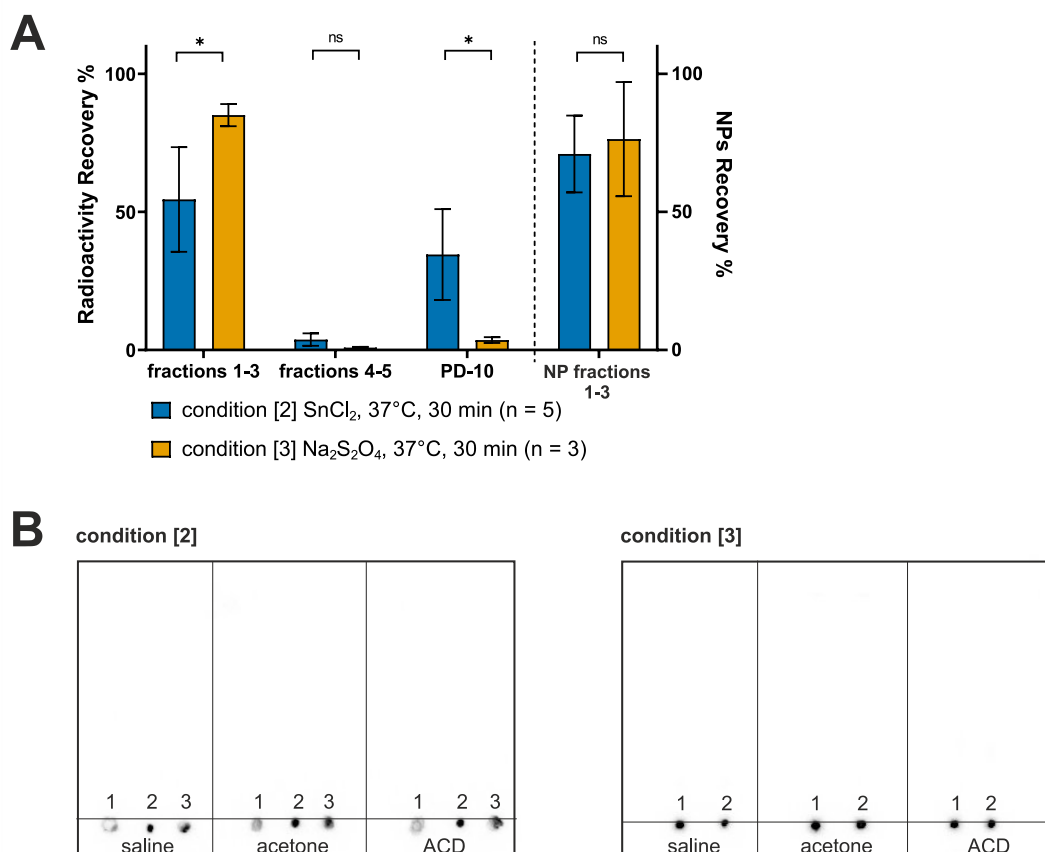


Figure 3 Characterization of the fractions following the purification of the reaction mixtures from labeling conditions [2] and [3] via PD-10 column. (A) Plot of radioactivity recovery/purification yield (left of dashed line) and nanoparticles recovery/purification yield (right of dashed line) after PD-10 column purification from radiolabeling conditions [2] and [3]. The NPs' recycling yield was calculated by dividing quantity of first three collected fractions after PD-10 column purification by the total amount of NPs used for radiolabeling. The levels of statistical significance are indicated as * $p < 0.05$, ns - no significance. (B) TLCs of the collected fractions after purification from conditions [2] and [3] using saline, acetone and ACD as developing solvents.

Additionally, TEM imaging was performed on all samples to verify that the purification process did not alter the NP morphology (Figure S11). While TLC analysis failed to differentiate the labeling success among these conditions, size-exclusion chromatography indicated higher radiolabeling efficiency of the $\text{Na}_2\text{S}_2\text{O}_4$ -based method over the one with SnCl_2 (Figure 3A). A difference has also been observed on dynamic SPECT/CT imaging and biodistribution studies in rats, after intravenous administration of a sample from condition [1] or [2].^[13,14] The sample prepared under condition [1], favoring HR- ^{99m}Tc formation, exhibited pronounced lung accumulation, followed by liver and spleen, due to the large particle size of colloids that trapped in the capillaries ($\varnothing$ 5 μm) of the lung (Figure 4A).

These results were consistent with the presence of radioactive impurities that remained undetected in the TLC analyses (Figure 3B).

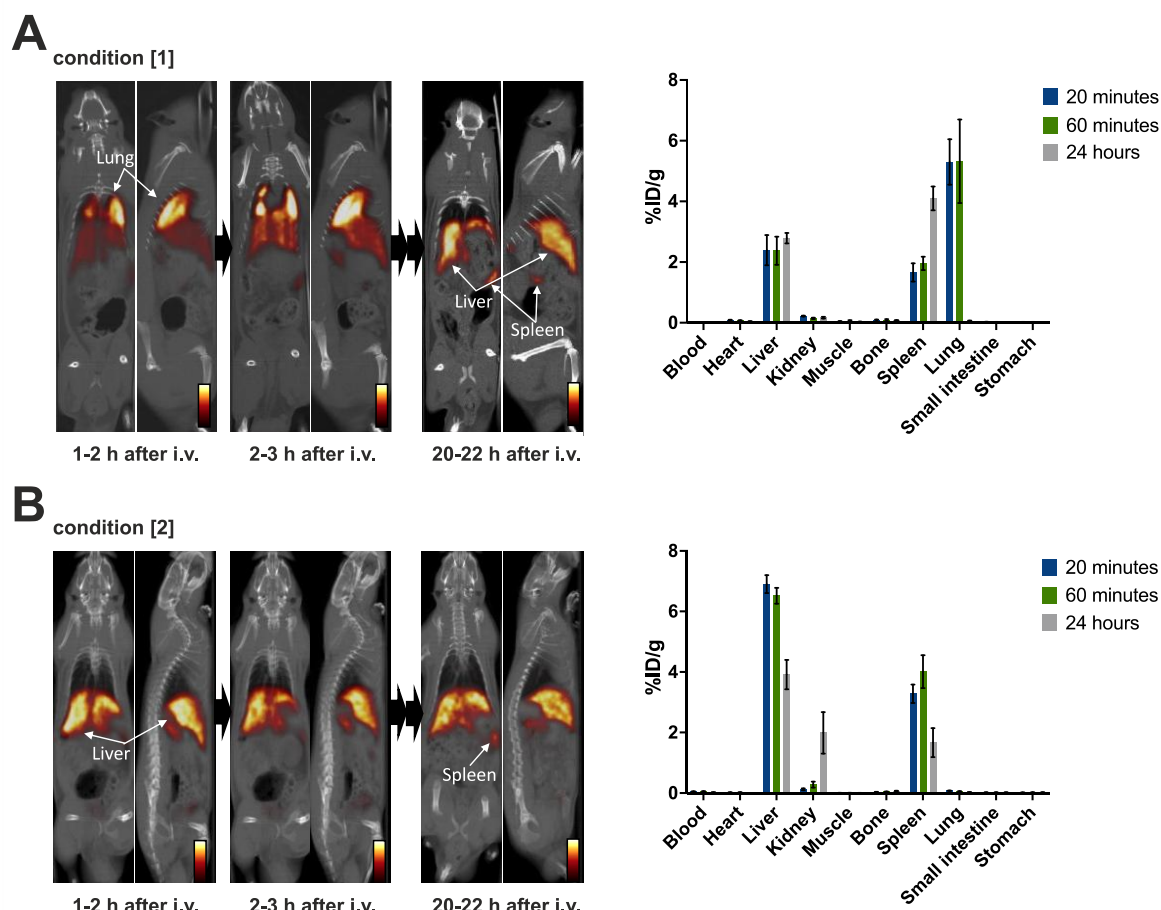


Figure 4 In vivo studies of ^{99m}Tc -NPs. Dynamic SPECT imaging fused with CT and biodistribution studies (20 min, 60 min and 24 h) in rats after intravenous administration of ^{99m}Tc -NPs radiolabeled in (A) experimental condition [1] and (B) [2] ([2] after purification with PD-10 column).

After 22 hours, the radioactivity slowly redistributed and was detected only in the liver and spleen. In contrast, ^{99m}Tc -NPs prepared under optimized condition [2] showed no colloid formation before and after purification, according to the DLS measurements (Figure 2D, Figure S10). Their observed distribution to the liver, spleen, and kidneys in SPECT/CT imaging in rats aligned with the expectations (Figure 4B). Overall, the obtained results indicate that the published TLC analysis could not sufficiently differentiate the radioactive species in the present system. This underscores the limitations of TLC for assessing the radiochemical purity of polymeric NPs and emphasize the critical need for complementary analytical techniques to accurately characterize NP formulations and their radiolabeled counterparts.

Conclusion

Our findings employing PLGA/PLA-PEG NPs as a model underscore the limitations of conventional TLC analysis for comprehensively assessing the radiochemical purity of ^{99m}Tc -labeled polymer NPs. The complex composition of these NPs, coupled with the challenges associated with analyzing polymeric materials using TLC, hinders the development of universally applicable methods. Furthermore, the potential formation of several possible radioactive impurities including colloids, which can significantly impact in vivo biodistribution, highlights the critical need for robust analytical techniques beyond TLC. To ensure accurate interpretation of in vivo data, a meticulous evaluation and validation of analytical methods is essential for each specific nanoparticle system.

Material and Methods

Nanoparticle Preparation

Core-shell nanoparticles were obtained by nanoprecipitation.^[24] For this purpose, PLA_{10K}-PEG_{5K}-COOH block copolymer was synthesized and poly(D,L-lactide-co-glycolide) (PLGA) (Resomer RG 502, acid-terminated, M_w 7000-17000, Sigma-Aldrich, St. Louis, USA) was covalently labeled with cyanine-5-amine (Lumiprobe GmbH (Europe), Hannover, Germany). CY-5-PLGA as the core material and PLA_{10K}-PEG_{5K}-COOH for the nanoparticle shell were adjusted in acetonitrile to a concentration of 10 mg mL⁻¹. The ratio of core to shell polymer was 3:7 (m/m). The organic phase was added dropwise into the 10-fold excess of stirring (930 rpm) aqueous phase, consisting of 10 % (v/v) Dulbecco's phosphate-buffered saline (DPBS) (Gibco™, Life Technologies, Paisly, UK) in Milli-Q water, and stirred for 3 h to remove the acetonitrile. Subsequently, the volume was concentrated by centrifugation at 3000 g for 15 min using Microseps Advance 30K centrifugal filters (molecular weight cut-off 30 kDa, Pall Corporation, New York, USA). Dynamic light scattering (DLS)-based measurements were carried out with a Malvern Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany) to characterize the nanoparticles regarding their size distribution and zeta potential. Nanoparticle concentrations were adjusted polymer content-based [mg mL⁻¹], with the amount of polyethylene glycol in each batch serving as a correlation factor.

Radiolabeling, TLC Analysis and Purification

To a mixture of 450 µL NPs (approximately 1 mg mL⁻¹) and 50 µL ^{99m}TcO₄⁻ (40 – 220 MBq in saline) 15 µL freshly prepared SnCl₂ solution (10 mg mL⁻¹ in 0.1 M citric acid or HCl) was added. The pH value of the solution was adjusted to 7 - 7.5 (Merck accurate pH indicator sticks pH 5 - 10), using 0.5 M NaHCO₃ and 0.5 M Na₂HPO₄. In case of Na₂S₂O₄, 100 µL of freshly prepared Na₂S₂O₄ solution (100 mg mL⁻¹ in water) was added to the mixture of NPs and ^{99m}TcO₄⁻, followed by the addition of 0.5 M NaHCO₃ to a final pH of 5. The solution was mixed gently and allowed to incubate either at room temperature or 37 °C for 30 min. An aliquot of 1 µL was removed afterwards and plotted on the starting line of each TLC (Silica gel 60 F254, Merck KGaA, Darmstadt, Germany). Saline, acetone, ACD (citrate dextrose solution, Merck KGaA, Darmstadt, Germany) and pyridine/acetic acid/water 3:5:1.5 (v/v) were used as developing solvents, respectively. The TLCs were exposed to Fujifilm Autoradiography film shortly followed by scanning on an autoradiography system (Amersham™ Typhoon™, GE Healthcare GmbH, Solingen, Germany). Public domain software ImageJ (version 1.53t) was used for the quantification. For staining with iodine vapor, the chamber was saturated with iodine vapor. The TLC plates were allowed to remain within the chamber until they had developed a light brown color over the entire plate.

For purification of radiolabeled NPs on size-exclusion columns, PD-10 desalting columns (bed volume 8.3 mL) with *Sephadex™ G-25 Medium* as fixed phase (GE Healthcare GmbH, Solingen, Germany) were preconditioned with 25 mL saline. The labeling mixture was loaded to the column,

and saline was added to allow the total loading volume to reach 2.5 mL. Saline was used as mobile phase and 5 × 1 mL were collected. Radioactivity and NP concentration of each fraction were measured in a dose calibrator and a microplate reader (Hidex Sense Beta Plus, Hidex Deutschland Vertrieb GmbH, Mainz, Germany), respectively. The typical radiochemical yield was calculated by dividing radioactivity of first three fractions collected after PD-10 column purification by the radioactivity before labeling incubation with decay correction. The NPs' recycling yield was calculated by dividing the quantity of first three collected fractions after PD-10 column purification by the total amount of NPs used for radiolabeling.

SPECT/CT Imaging and Biodistribution Studies

All animal experiments were approved by the Ethics Commission of the government of Lower Franconia, Bavaria, Germany (approval number RUF-55.2.2-2532-2-1765-21) and were conducted strictly according to the Guide for the Care and Use of Laboratory Animals^[25] and the ARRIVE guidelines.^[26] Anesthesia was induced in healthy male Wistar rats (n = 1 for each SPECT/CT imaging, n = 3 for biodistribution study at each time point, weighing 350-560 g, Charles River Laboratories, Research Models and Services, Japan) by using 5 % isoflurane to induce anesthesia and maintained during the whole experiment with 2 % isoflurane. SPECT/CT imaging was obtained according to a previous described protocol.^[27] In brief, SPECT imaging in rats was obtained using a dedicated small animal SPECT/CT system (MILabs B.V., Houten, The Netherlands). ^{99m}Tc-NPs (100 MBq) were injected via the tail vein of the animals. A SPECT scan was initiated with acquisition in list-mode 1 and 20 h post-injection. The obtained SPECT images were analyzed with the public domain tool AMIDE imaging software (A Medical Imaging Data Examiner, version 1.01). For the biodistribution studies, ^{99m}Tc-NPs (0.5 to 1,3 MBq each animal, n = 3 each time point) were administered via the tail vein. The animals were euthanized at 20 min, 60 min and 24 h after radiotracer administration. The organs of interest were harvested and weighed, followed by tissue counting with a γ-counter (2480 Automatic Gamma Counter WIZARD2, PerkinElmer LAS GmbH, Rodgau, Germany). The results are expressed as percentage of injected dose per gram of tissue (%ID/g).

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Supplementary Information

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K. S. and X. C. contributed equally to this work (shared first authors). K. S. and X. C. designed and performed the research, analyzed the data and wrote the manuscript. K. S. performed the experiments related to nanoparticle preparation and characterization and conducted TLC experiments with nanoparticles and polymers. X. C., T. S. and A.-P. A.-L. conducted the radiolabeling experiments and the animal studies. J. L. synthesized the PLGA-CY-5. T. H. and A. G. supervised the project, provided scientific input during the complete process, supported the manuscript preparation and reviewed the final manuscript.

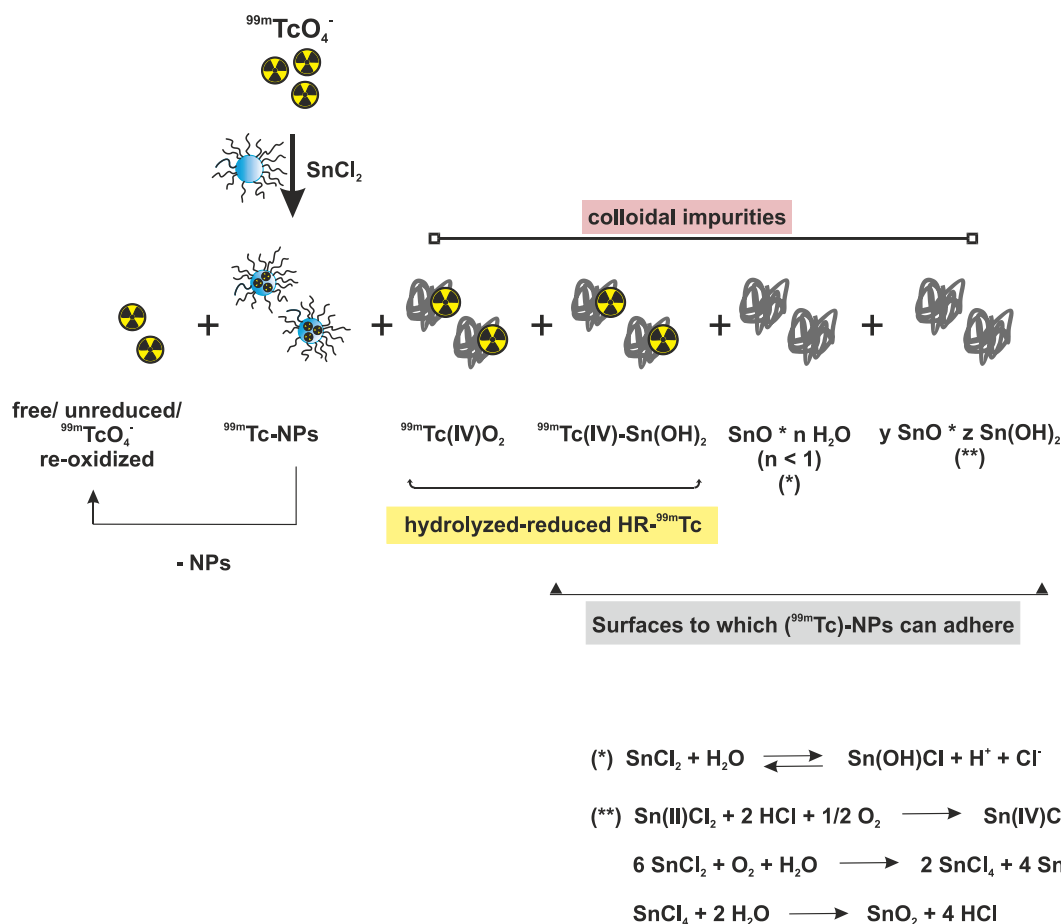


Figure S 1 Schematic depiction of impurities. Potential impurities occurring in the reaction vessel^[1,2] and the related tin chemistry. Tin(II) chloride can be hydrolyzed in aqueous solution to an insoluble tin hydroxide salt. In addition to hydrolysis to tin oxide ($\text{SnO}_2 \cdot n\text{H}_2\text{O}$), tin chloride can also be oxidized in the presence of atmospheric oxygen. The resulting tin tetrachloride (SnCl_4) is hydrolyzed in aqueous solution.^[3]

Table A

Labeling procedure	
1)	450 μL of nanoparticles (1.1 mg/ml, pH 5) were added to the reaction vessel
2)	450 μL of $[^{99\text{m}}\text{TcO}_4]^-$ solution in saline were added
3)	A defined volume of SnCl_2 solution (2 mg/ml, solubilized in 10 mM HCl)
	[1] 100 μL SnCl_2 solution was added [2] 20 μL SnCl_2 solution was added
4)	The reaction mixture was stirred at 500 rpm for 1.5-2 h at room temperature

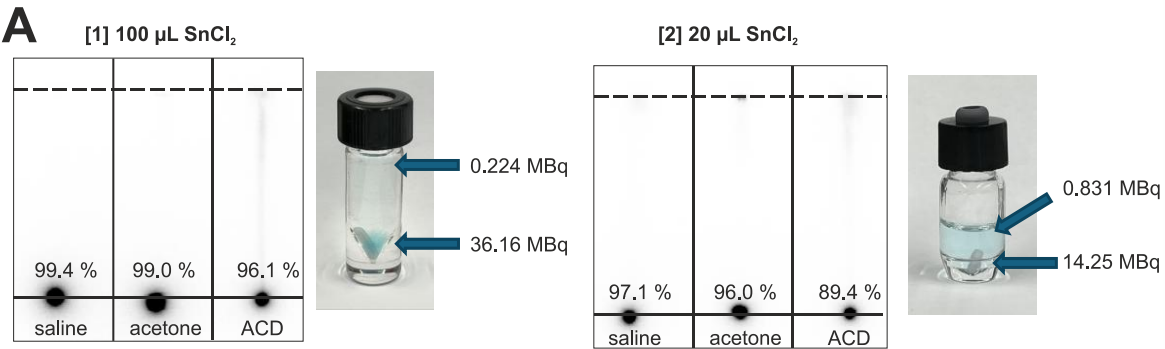


Table B

Labeling procedure	
1)	450 μL of $[^{99\text{m}}\text{TcO}_4]^-$ solution in saline were added to the reaction vessel
2)	Vacuum was applied to the reaction vessel and it was subsequently purged with nitrogen
	A defined volume of SnCl_2 solution (1 mg/ml, solubilized in 100 mM HCl)
3)	[3] 60 μL SnCl_2 solution was added [4] 100 μL SnCl_2 solution was added
4)	The reaction mixture was stirred at 500 rpm for 5 min at room temperature
5)	10 μL of 0.5 M NaHCO_3 solution and 450 μL of nanoparticles (1.1 mg/ml) were added
6)	The reaction mixture was stirred at 500 rpm for 20 min at room temperature

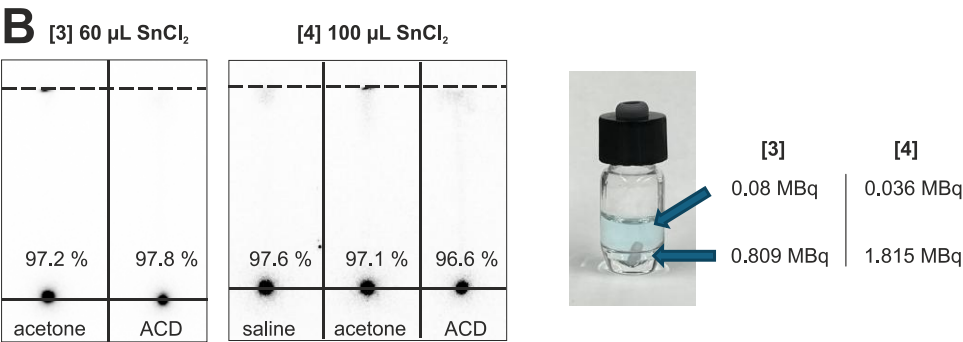
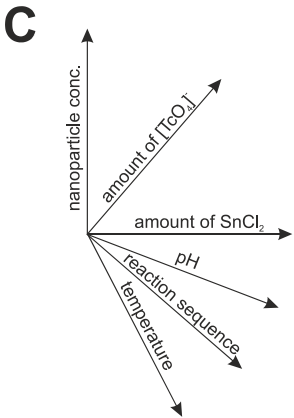


Figure S 2 Radiolabeling studies on CY-5-labeled PLGA/PLA-PEG nanoparticles (NPs). Exemplary representation of labeling protocols (Table A and B) with $^{99\text{m}}\text{TcO}_4^-$ and SnCl_2 as reducing agent, and (A) and (B) the resulting batches and their TLC analyses using saline, acetone and ACD solution as developing solvents. (C) Main factors influencing the labeling efficiency of NPs.^[4–8]

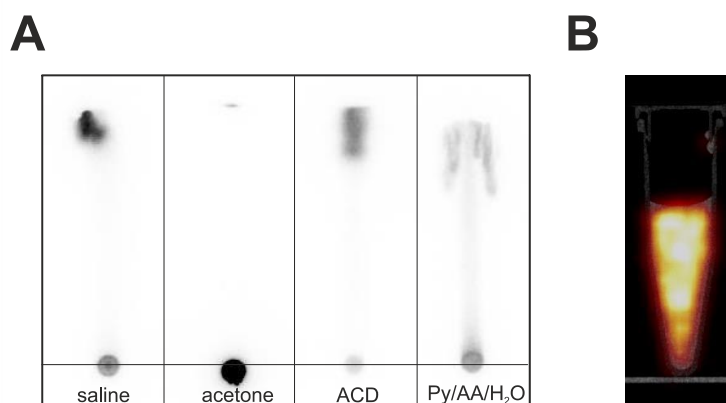


Figure S 3 Reaction mixture of NPs, [TcO₄⁻] and SnCl₂ solubilized in citric acid and reacted at room temperature for 30 min. (A) TLCs of the reaction mixture. Saline, acetone, ACD and Py/AA/H₂O were used as developing solvents. (B) SPECT/CT fusion imaging of the reaction mixture.

Extended Experimental Section

Materials

All materials and reagents described below were purchased from Sigma Aldrich (Taufkirchen, Germany or St. Louis, USA) unless otherwise stated. The ultrapure water used for the experiments was treated with a Milli-Q EQ 7000 system (Merck, Darmstadt, Germany) equipped with Milli-Q Biopak filter (Merck, Darmstadt, Germany) and freshly taken every day. It is referred to as Milli-Q water in the following.

Fluorescence Labeling of PLGA with CY-5

Poly(D,L-lactide-co-glycolide) (PLGA) (Resomer RG 502, carboxylic acid-terminated, M_w 7000-17000, Sigma-Aldrich, St. Louis, USA) was fluorescently labeled by covalent linkage with cyanine-5-amine (Lumiprobe GmbH (Europe), Hannover, Germany). In brief, carboxylic acid-terminated PLGA (1 equiv.) was dissolved together with cyanine-5-amine (0.1 equiv.) in anhydrous *N,N*-dimethylformamide (DMF). The carboxylic acid group was activated by the addition of (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (2 equiv.) and *N,N*-diisopropylethylamine (DIPEA) (4 equiv.). The reaction mixture was stirred overnight at room temperature (RT) under light protection. To remove the excess of free dye, the polymer was precipitated several times in ice-cold diethyl ether until the supernatant was no longer blue. The product was dried under the nitrogen flow and characterized by FTIR. FTIR spectra were recorded on an Agilent Technologies Cary 630 FTIR (Agilent, Santa Clara, USA) with 32 scans, 8 cm^{-1} resolution (Figure S4). The purity was confirmed by high performance liquid chromatography (HPLC) analysis (Agilent Technologies 1260 Infinity II, Santa Clara, USA). For this, a C18 column (Kinetex 2.6 μm , EVO C18 100 Å, 150 x 4.6 mm) was used, that was heated to 40 °C. 0.05 % trifluoroacetic acid in acetonitrile [A] and 0.05 % trifluoroacetic acid in Milli-Q water [B] were employed as the mobile phases for the gradient elution at a flow rate of 1.0 mL min^{-1} ([A] 10 to 95 % - 0-10 min/ [A] at 95 % - 10-13 min/ [A] 95 to 10 % - 13-15 min/ [A] at 10 % - 15-20 min). Fluorescence detection was performed at an excitation wavelength λ 647 nm and an emission wavelength of λ 665 nm. Emission spectra were recorded in the peaks (scan range: λ 630 nm to λ 680 nm, step: 5 nm). 2 μL sample volume (dissolved in acetonitrile, polymers diluted to a concentration of 3.3 μM , CY-5-amine diluted to a concentration of 0.2 μM) were applied in each run. To double-prove the elution behavior of polymer free from unreacted CY-5-amine and polymer fragments, PLGA-CY-5 NPs were produced in Milli-Q water from the analyzed polymer batch. They were purified with a PD-10 column (SephadexTM G-25M, cytiva, Buckinghamshire, UK). After purification, the samples were freeze-dried (Alpha 2-4 LSCplus, Christ, Osterode am Harz, Germany), dissolved in DCM and TLC analyses were performed.

Block Copolymer Synthesis and Characterization

COOH-PEG_{5k}-PLA_{10k} block copolymer was synthesized via ring-opening polymerization of the cyclic lactone 3,6-dimethyl-1,4-dioxane-2,5-dione with the organocatalyst 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) at room temperature. COOH-PEG_{5k}-OH (Jenkem Technology, Plano, USA, M_n 5000 $\pm$ 500 g mol⁻¹, as stated on certificate of analysis) was used as macroinitiator for the reaction.^[9,10] In brief, polyethylene glycol (PEG) macroinitiator (1 equiv.) was reacted with 3,6-dimethyl-1,4-dioxane-2,5-dione (70 equiv.) and DBU (3.5 equiv.) in dichloromethane (DCM) as solvent at RT. After 1 h the reaction was quenched with benzoic acid in excess (17.5 equiv.). The resulting block copolymer was precipitated in ice-cold diethyl ether, filtered, and dried under the nitrogen flow. Identity and purity of the block copolymer was verified by ¹H-NMR spectra recorded on a Bruker Avance III HD 400 operating at 400 MHz; the block copolymer was dissolved in deuterated chloroform (CDCl₃).^[11] Furthermore, the molecular weight of the synthesized polymer was determined from the ¹H-NMR spectra as previously described by our group.^[12] Topspin 4.3.0 software was used to evaluate the NMR spectra (Figure S5).

NP Characterization

Dynamic light scattering (DLS)-based measurements (particle size distribution, zeta potential, stability data) were carried out with a Malvern Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany) equipped with a λ 633 nm He-Ne laser operating at an angle of 173°. The software used for data collecting and processing was Zetasizer software version 7.12 (Malvern Instruments, UK). To determine the size distribution, the concentrated samples were diluted 1:20 with 10 % DPBS in Milli-Q water (10 % Dulbecco's phosphate-buffered saline (DPBS) (v/v)). 90 μ L sample volume were measured in micro-UV-cuvettes (Carl Roth, Karlsruhe, Germany) at a controlled temperature of 25 °C (120 sec equilibration time). Three repetitions with 11 runs of 10 sec duration were performed for each sample. The data was analyzed with the software-integrated analysis model for "general purposes (normal resolution)". For zeta potential measurements, the samples were diluted 1:200 with 10 % PBS in Milli-Q water. The samples were measured in folded capillary zeta cells (Malvern, Herrenberg, Germany) at 25 °C. Three repetitions with at least 60 runs were performed for each sample. The data was analyzed by "auto mode" (Figure S6).

Iodine Assay

The PEG amount was quantified via an iodine assay adapted from Childs^[13] as previously published by our group.^[9] In brief, poly(ethylene glycol) monomethyl ether with an average molecular weight of 5000 g mol⁻¹ (mPEG_{5k}) was used as calibration standard in aqueous solutions (0 – 20 μ g mL⁻¹). 140 μ L of standard dilutions and NPs as samples were pipetted into a 96-well plate and incubated for 10 min under light exclusion with 60 μ L of a solution composed of two parts of 5 % barium chloride (m/v) (Merck, Darmstadt, Germany) dissolved in 1 M HCl and one part 0.05 M iodine

solution (VWR, Lueven, Belgium). The absorbance was measured at a wavelength of λ 535 nm on a FLUOstar Omega plate-reader (BMG Labtech, Ortenberg, Germany) (Figure S7).

Polymer Content-Based NP Quantification

To determine the NP concentration in units of $[\text{mg mL}^{-1}]$ based on polymer content, three independent batches of NPs were prepared in pure Milli-Q water according to the procedure described for NP preparation. The PEG content of the NPs was determined via colorimetric iodine assay.^[13] Furthermore, 100 μL of each NP batch were freeze-dried (Alpha 2-4 LSCplus, Christ, Osterode am Harz, Germany) and the remaining dry polymer was accurately weighed to determine the mass-concentration $[\text{mg mL}^{-1}]$ (mass-based quantification). Assuming constant amounts of PEG between batches of the same NP type, the mass concentration was correlated with the PEG content, using the correlation factor k as previously described by our group (Figure S7).^[9]

This approach allowed for the polymer content-based quantification of the NPs based on the determined PEG content as shown in equation 1:

$$c(\text{NP}) \left[\frac{\text{mg}}{\text{mL}} \right] = c(\text{PEG}) \left[\frac{\mu\text{g}}{\text{mL}} \right] \cdot k$$

eq. 1

Transmission Electron Microscopy

For sample preparation 9 nm carbon film coated 3.5 nm Cu TEM grids (400 mesh) (Plano GmbH, Wetzlar, Germany) were hydrophilized in the oxygen plasma. 5 μL of the respective NP dispersion were applied to the grids for 5 sec and in the last application cycle for 1 min. The application procedure was repeated up to four times depending on the NP concentration in the dispersion. Afterwards, the excess samples were removed carefully with a filter paper. The copper-grids were washed with Milli-Q water and exposed for 30 s to an aqueous dilution of uranyl acetate (1 %). A stack of images at different positions was recorded on JEM-2100F Field emission electron microscope (Jeol, Freising, Germany) at 20,000-fold magnification using the SerialEM software. The images were analyzed using Fiji^[14] software (Figure S11).

In Vitro Stability and Challenge Tests

The stability of $^{99\text{m}}\text{Tc}$ -NPs was assessed both in vitro, in saline, and in co-incubation with human serum. Specifically, 100 μL of the freshly collected fraction after PD-10 purification was combined with an equal volume of either saline or human serum, followed by an incubation at 37 °C for 1 – 4 h. Challenge tests were conducted similarly, with 100 μL of the freshly collected fraction after PD-10 purification being incubated with either 10 mM DTPA or cysteine solution at 37° C for 1 – 4 h. At each hour interval, an aliquot was withdrawn and subjected to TLC analysis using saline, acetone, and

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ACD solution as the developing solvents. Subsequently, the TLC plates were examined using an autoradiography system, following the aforementioned protocol (Figure S12).

Calculation of the Distance between Solvents and Polymers in the “Hansen Space”

The solubility of the polymeric components of the NPs in the solvents used as mobile phase for TLC analyses was estimated based on their Hansen solubility parameters. The concept of the solubility parameter (δ) was first introduced by Hildebrand, defined as square root of the quotient of cohesion energy (ΔH_v) and the molar volume (V_M),^[15] as shown in equation 2:

$$\delta = \left(\frac{\Delta H_v - RT}{V_M} \right)^{1/2}$$

eq. 2

The concept was later expanded by Hansen by splitting the Hildebrand solubility parameter into three solubility parameters derived from different types of cohesive forces (equation 3):

$$\delta = (\delta_d^2 + \delta_p^2 + \delta_h^2)$$

eq. 3

δ_d , referred to as dispersion parameter, corresponds to the London interactions that the analyzed substance can enter. δ_p , referred to as polar parameter, correlates with the polar groups, and δ_h , referred to as hydrogen bonding parameter, represents hydrogen bonding forces. Solvents and solutes with similar Hansen solubility parameters are expected to form thermodynamically more stable solutions.^[15]

The solubility of the polymers in the used solvents can therefore be estimated by calculating their distance (D) in the so-called “Hansen space” according to equation 4, where the parameters of the solvents are denoted by S and the parameters of the polymers by P :

$$D = (4 * (\delta_{dS} - \delta_{dP})^2 + (\delta_{pS} - \delta_{pP})^2 + (\delta_{hS} - \delta_{hP})^2)^{1/2}$$

eq. 4

The parameters and their distances are listed in Figure S9. The following simplifications were made:

- (1) The PLA-PEG block copolymer was split into PLA and PEG.
- (2) PLGA was analyzed without the covalently coupled CY-5.
- (3) The solvents were not analyzed as a mixture.

Statistics

The results of the collected fractions after PD-10 column purification are displayed as the mean $\pm$ SD (Figure 3). Multiple group comparisons were performed using analysis of variance (two-way ANOVA).

A *p*-value of less than 0.05 was assumed to be statistically significant. Statistical analysis was performed with GraphPad Prism (version 9.4.1 GraphPad Software, San Diego, USA).

Software

The software used for the different experiments is listed below.

TopSpin 4.1.4 software (Bruker Corporation/Billerica/MA/USA, URL: <https://www.bruker.com/en/products-and-solutions/mr/nmr-software/topspin.html>) was used for NMR processing and analysis.

Origin 2022b software (OriginLab Corporation/Northampton/Ma/ USA. URL: <https://www.originlab.com/2022b>) was used for data plotting and analyzing.

R software (R Core Team (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>) was used for data plotting and statistical analysis.

Fiji software^[14] for TEM image processing.

PerkinElmer ChemOffice Suite 2018 was used to draw chemical structural formula and chemical reaction equations.

Public domain software ImageJ (version 1.53t) was used for the quantitative evaluation of the TLC analysis.

Prism GraphPad (version 19.4.1) was used for the plotting and statical analysis of elution fractions after PD-10 purification.

AMIDE imaging software (A Medical Imaging Data Examiner, version 1.01) was used to analyze the SPECT images.

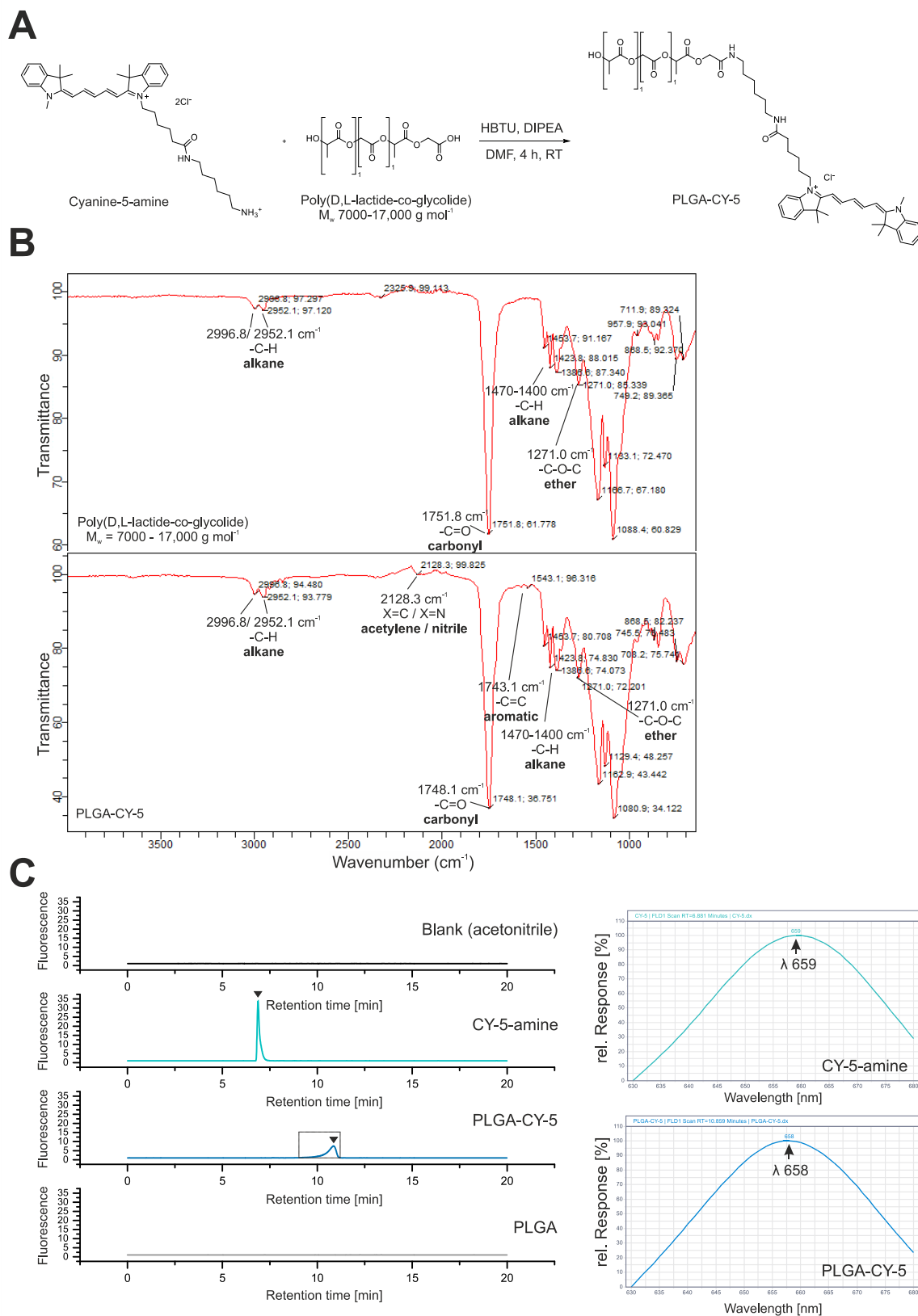
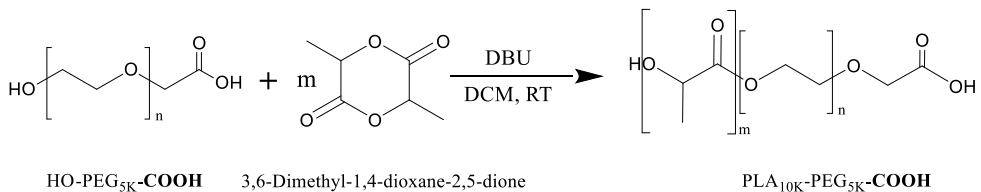


Figure S 4 Synthesis and characterization of PLGA-CY-5. (A) Reaction equation of fluorescence labeling of PLGA with CY-5. (B) FTIR spectra of poly(D,L-lactide-co-glycolide) (PLGA, M_n 7000-17,000 g mol⁻¹) and PLGA-CY-5. Peaks for acetylene/nitrile (2128.3 [cm⁻¹] ν (X=C/ X=N)) and aromatic groups (1743.1 [cm⁻¹] ν (-C=C)) were found in the spectrum of PLGA-CY-5. (C) HPLC chromatograms of acetonitrile (blank), CY-5-amine (sample), PLGA-CY-5 (sample) and PLGA (reference) and emission spectra of the peaks (CY-5-amine:

6.879 min (retention time)/ PLGA-CY-5: 10.856 min (retention time)) to prove the purity of the CY-5-labeled polymer from unreacted CY-5-amine and the success of the coupling reaction. The width (retention time: 8.5 - 11.5 min) of the PLGA-CY-5 peak serves as good indicator for the broad molecular weight distribution of the polymer (M_w 7000-17000) and the variation in retention times resulting from this.

A



B

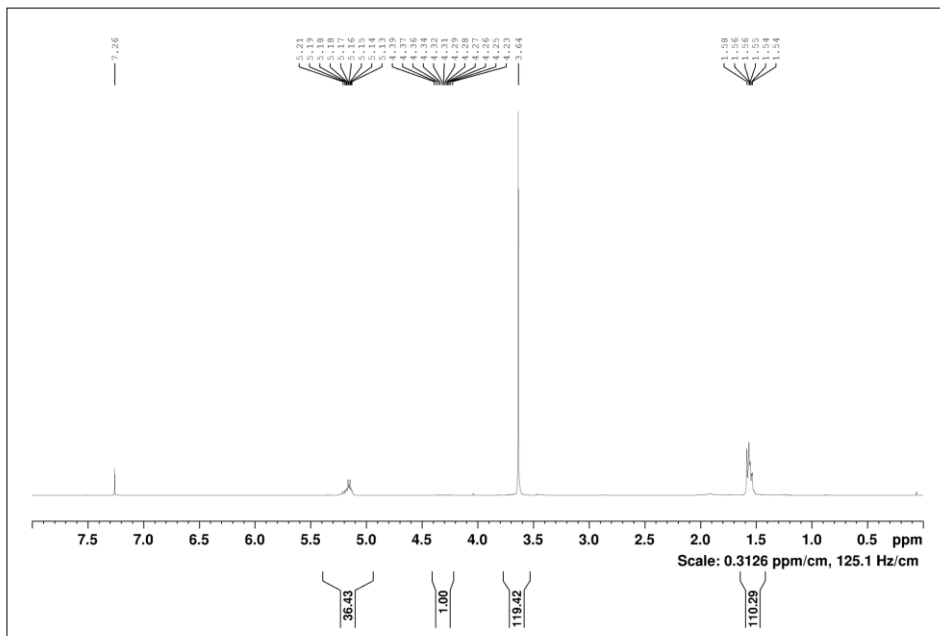


Figure S 5 Block copolymer synthesis and characterization. (A) Reaction equation for the synthesis of PLA_{10K}-PEG_{5K}-COOH block copolymer through ring opening polymerization of cyclic lactone 3,6-dimethyl-1,4-dioxane-2,5-dione with poly(ethylene glycol) chains as macroinitiators for the reaction. (B) ¹H-NMR spectrum (CDCl₃, 400 MHz) of PLA_{10K}-PEG_{5K}-COOH block copolymer. **δ (ppm):** 5.17 ppm (m, -C(CH₃)H-), 4.31 ppm (m, -OCH₂CH₂-O(CO)-), 3.64 ppm (m, HOOCCH₂CH₂-), 1.56 ppm (m, -C(CH₃)H-).

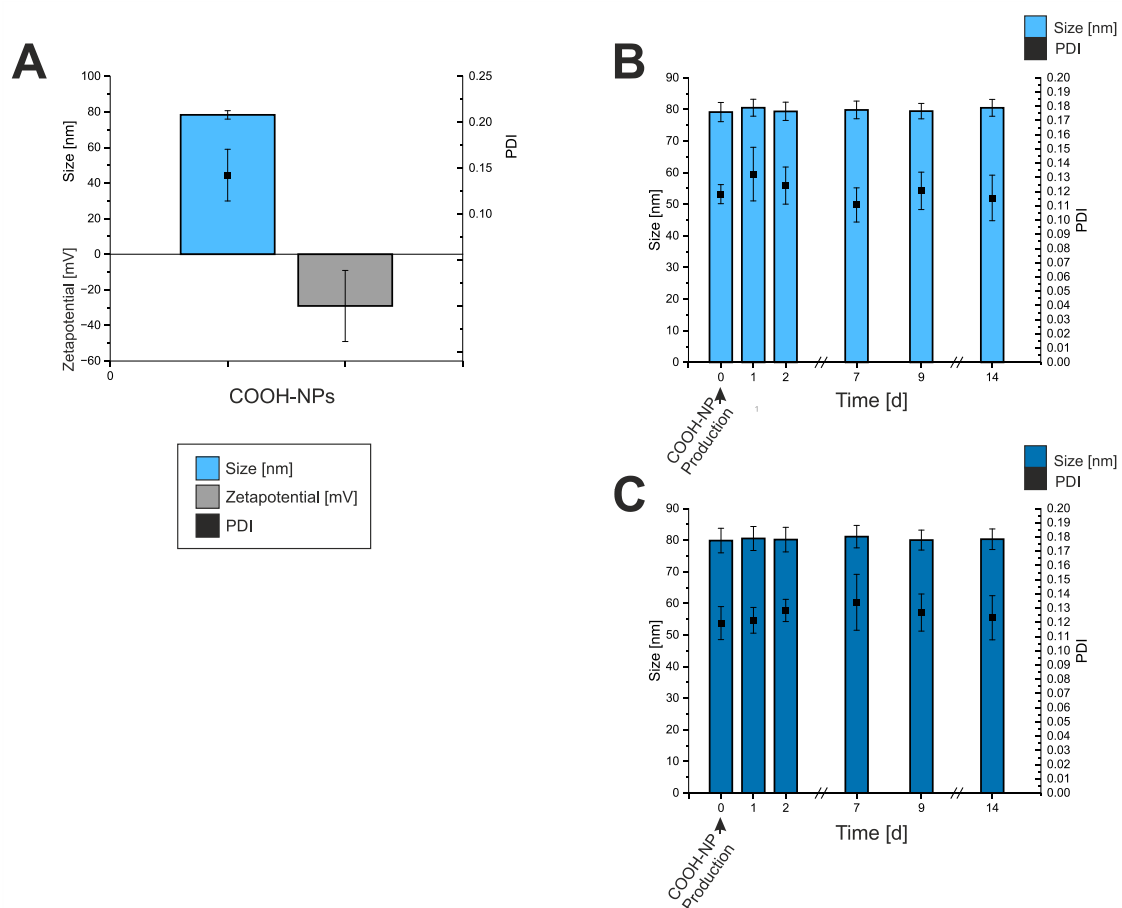


Figure S 6 NP characterization. (A) NP data on hydrodynamic diameters, polydispersity index (PDI) and zeta potential obtained from DLS measurements. Results represented as mean $\pm$ SD ($N = 3$; $n = 3$). (B) Stability data (hydrodynamic diameter and PDI) of COOH-NPs at 22 °C over 14 d. Results represented as mean $\pm$ SD ($N = 3$; $n = 3$). (C) Stability data of COOH-NPs at 2-8 °C over 14 d. Results represented as mean $\pm$ SD ($N = 3$; $n = 3$). The data indicate that the NPs were stable in size over 14 d under both storage conditions.

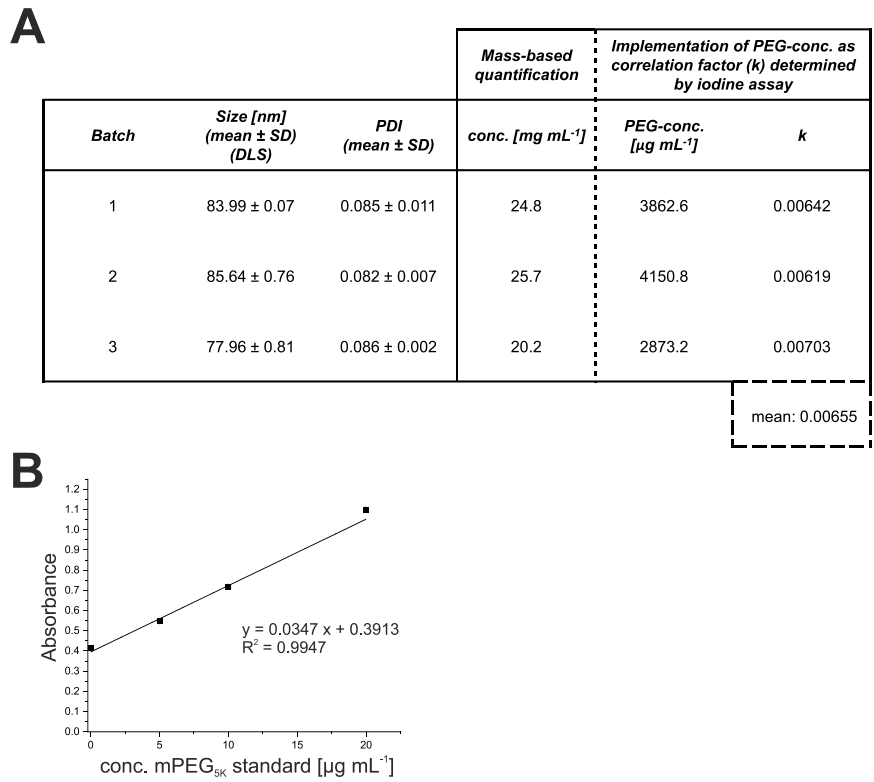
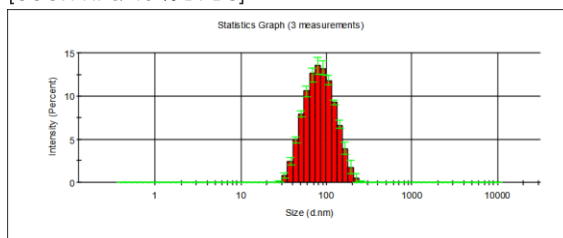


Figure S 7 Polymer content-based NP quantification. (A) Production and characterization of three independent NP batches (COOH-NPs) in pure Milli-Q water to determine correlation factors (*k*) and calculate the polymer content-based NP concentration. Results represented as mean ± SD (n = 3). (B) Calibration curve of the iodine assay against mPEG_{5K} standard. Results represented as mean ± SD (n = 3).

A

[COOH-NPs/ 10 % DPBS]



[COOH-NPs/ acetone]

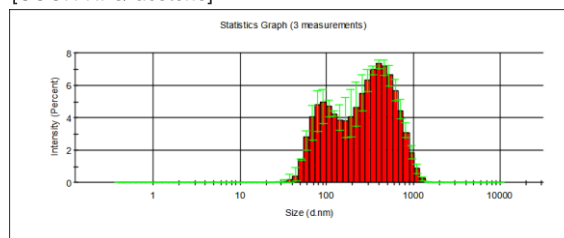
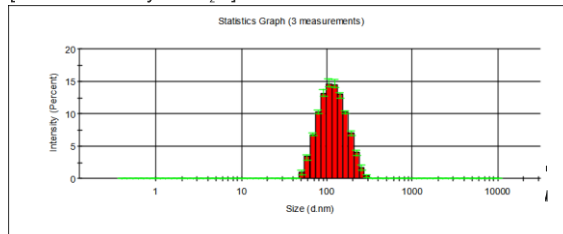
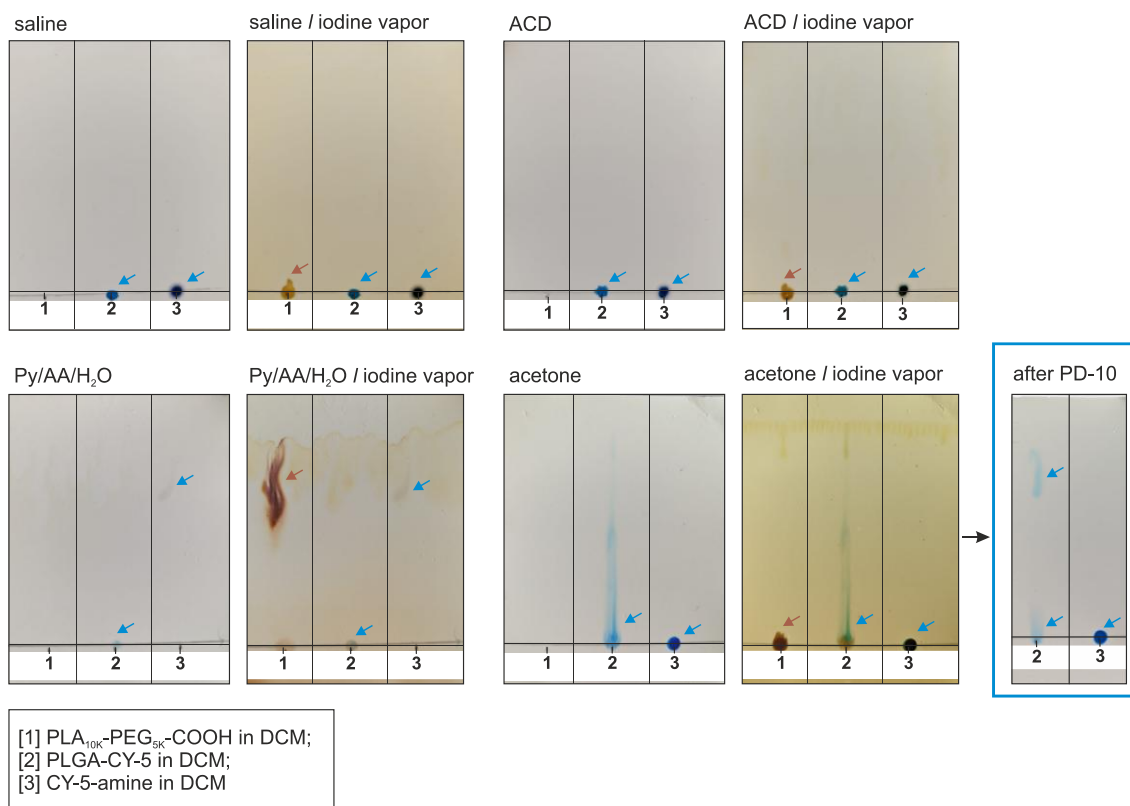
[COOH-NPs/ Py/AA/H₂O]**B**

Figure S 8 (A) DLS raw data recorded with a Malvern Zetasizer Nano ZS (n = 3). COOH-NPs/ 10 % DPBS: sample of unlabeled NPs diluted 1:20 with 10 % DPBS in Milli-Q water as reference. COOH-NPs/ acetone: sample of unlabeled NPs diluted 1:4 with acetone and subsequently diluted 1:20 with 10 % DPBS in Milli-Q water. COOH-NPs/ Py/AA/H₂O: sample of unlabeled NPs diluted 1:4 with a mixture of pyridine: acetic acid: water (3:5:1.5, (v/v)) and subsequently diluted 1:20 with 10 % DPBS in Milli-Q water. **(B) TLCs of polymers and CY-5-amine before and after staining with iodine vapor.** [3] CY-5-amine served as control. The TLC analysis with acetone as solvent was repeated after purification of the PLGA-CY-5 with a PD-10 column to verify that the polymer sample was not contaminated with unreacted CY-5-amine or polymer fragments (lactides). The elution behavior of the polymer did not differ before and after purification.

Table A

Component	δ_d	δ_p	δ_h
PEG (#1)	20.3	9.6	6.0
PLA (#1)	19.8	4.0	6.7
PLGA (#1)	17.4	9.1	10.5
acetonitrile (#2)	15.3	18.0	6.1
acetone (#3)	15.7	5.3	11.7
pyridine (#2)	19.0	8.8	5.9
acetic acid (#3)	14.5	8.0	13.5

Table B

Distance D			
	PEG	PLA	PLGA
acetonitrile	13.1	16.7	10.8
acetone	11.6	9.7	5.2
pyridine	2.7	5.1	5.6
acetic acid	13.9	13.2	6.6

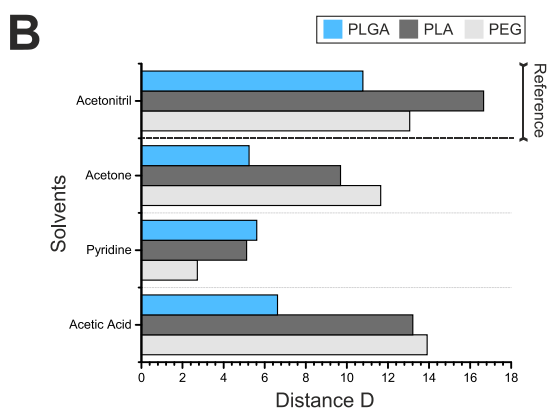


Figure S 9 Hansen solubility parameters of polymers and solvents and their distances in the “Hansen space”. (#1 obtained from Maslanka Figueroa et al.,^[16] #2 obtained from Tang et al.,^[17] #3 obtained from Bordes et al. 2010^[15]). (B) The solubility of the individual polymers in the mobile phase was calculated as an estimate of the distance D between solvent and solute in the “Hansen space”. In direct comparison to acetonitrile, which was used as organic phase in the NP production process, the polymers showed comparable or better solubility in the organic solvents and acetic acid.

Unlabeled NPs (reference)

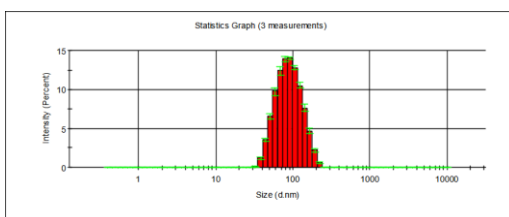
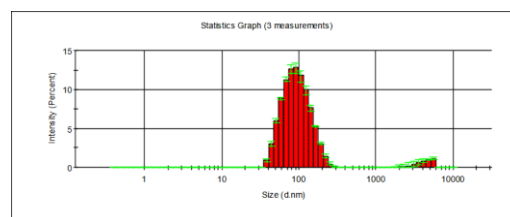
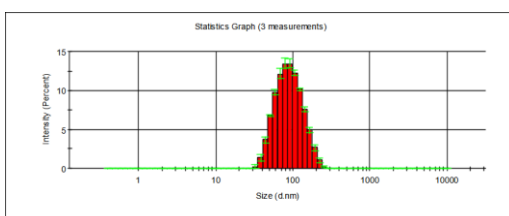
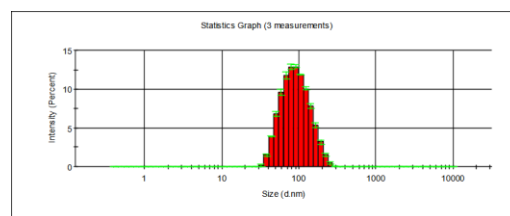
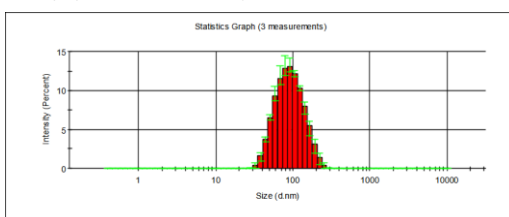
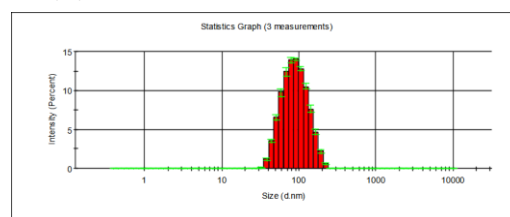
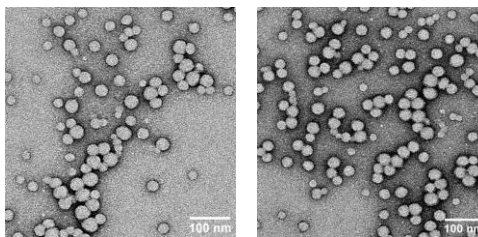
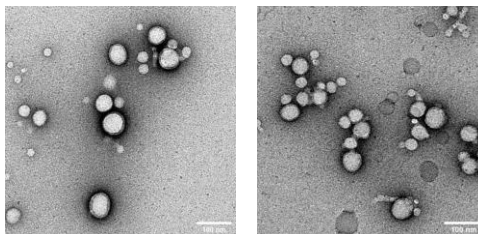
 $^{99m}\text{Tc(IV)}$ -NPs after labeling, SnCl_2 , RT $^{99m}\text{Tc(IV)}$ -NPs after labeling, SnCl_2 , 37 °C $^{99m}\text{Tc(IV)}$ -NPs after PD-10 column purification, SnCl_2 , 37 °C $^{99m}\text{Tc(IV)}$ -NPs after labeling, $\text{Na}_2\text{S}_2\text{O}_4$  $^{99m}\text{Tc(IV)}$ -NPs after PD-10 column purification, $\text{Na}_2\text{S}_2\text{O}_4$ 

Figure S 10 DLS raw data recorded with a Malvern Zetasizer Nano ZS (n = 3). Representative measurements of a NP sample before labeling with $^{99m}\text{Tc(IV)}$ (*unlabeled NPs (reference)*), after labeling at room temperature using SnCl_2 as reducing agent (*$^{99m}\text{Tc(IV)}$ -NPs after labeling, SnCl_2 , RT*), after labeling at 37 °C using SnCl_2 as reducing agent, before (*$^{99m}\text{Tc(IV)}$ -NPs after labeling, SnCl_2 , 37 °C*) and after purification using a PD-10 column (*$^{99m}\text{Tc(IV)}$ -NPs after PD-10 column purification, SnCl_2 , 37 °C*), after labeling using $\text{Na}_2\text{S}_2\text{O}_4$ as reducing agent before (*$^{99m}\text{Tc(IV)}$ -NPs after labeling, $\text{Na}_2\text{S}_2\text{O}_4$*) and after purification using a PD-10 column (*$^{99m}\text{Tc(IV)}$ -NPs after PD-10 column purification, $\text{Na}_2\text{S}_2\text{O}_4$*).

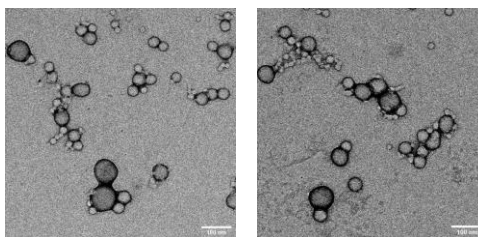
Unlabeled NPs (as reference)



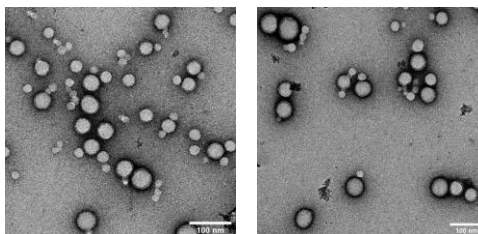
^{99m}Tc -NPs, [1] SnCl_2 , RT, 30 min



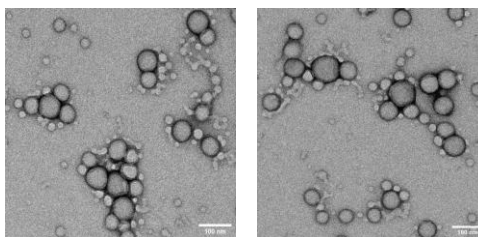
^{99m}Tc -NPs, [2] SnCl_2 , 37 °C, 30 min, after labeling



^{99m}Tc -NPs, [2] SnCl_2 , 37 °C, 30 min, after PD-10 column purification



^{99m}Tc -NPs, [3] $\text{Na}_2\text{S}_2\text{O}_4$, 37 °C, 30 min, after labeling



^{99m}Tc -NPs, [3] $\text{Na}_2\text{S}_2\text{O}_4$, 37 °C, 30 min, after PD-10 column purification

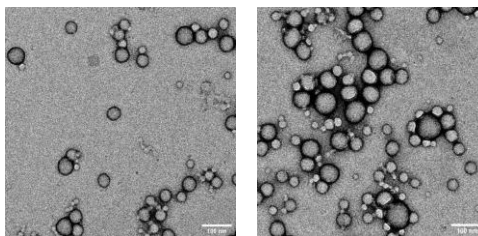


Figure S 11 TEM images of unlabeled NPs (as reference) and NPs labeled under different conditions, before and after purification via PD-10 column (scale bar: 100 nm).

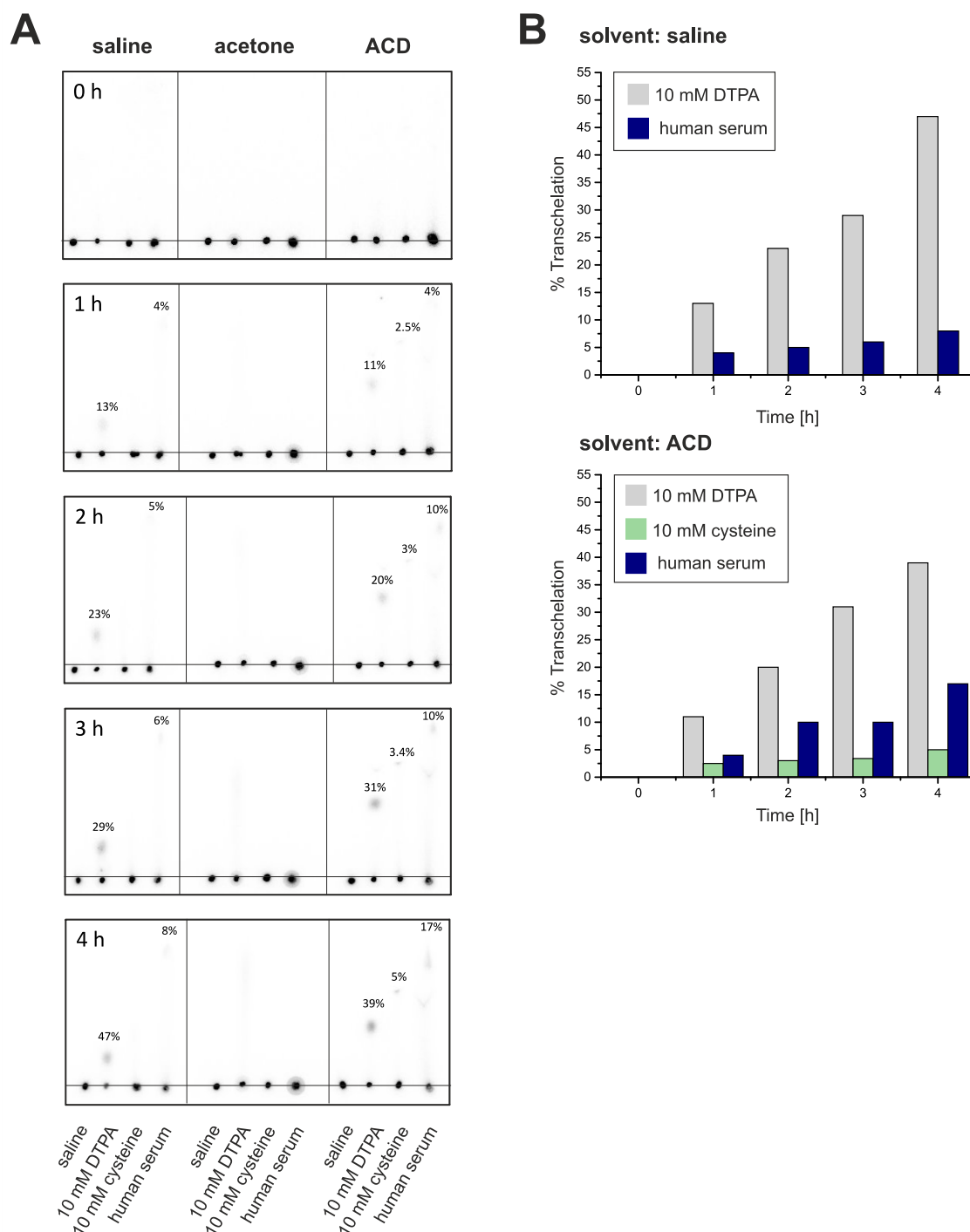


Figure S 12 Results of in vitro stability and challenge tests. In vitro stability and challenge tests with saline, 10 mM DTPA, 10 mM cysteine, and human serum were performed. The results indicate that the ^{99m}Tc -NPs were relatively stable in human serum, with the majority radioactivity remain bound to the NPs after 4 h of co-incubation. According to published physiologically based pharmacokinetic (PBPK) models, comparable polymer NPs circulate in the bloodstream for several hours after intravenous administration and accumulate particularly in the organs of the reticuloendothelial system.^[18] (A) TLCs of ^{99m}Tc -NPs mixed with 1) saline, 2) 10 mM DTPA, 3) 10 mM cysteine, 4) human serum, incubated at 37 °C for 1 - 4 h. Saline, acetone and ACD were used as developing solvents. ^{99m}Tc transchelated in DTPA, cysteine or serum components is eluted with aqueous solvents (saline and ACD), but not with organic solvents (acetone). (B) Transchelation shown as percentage.

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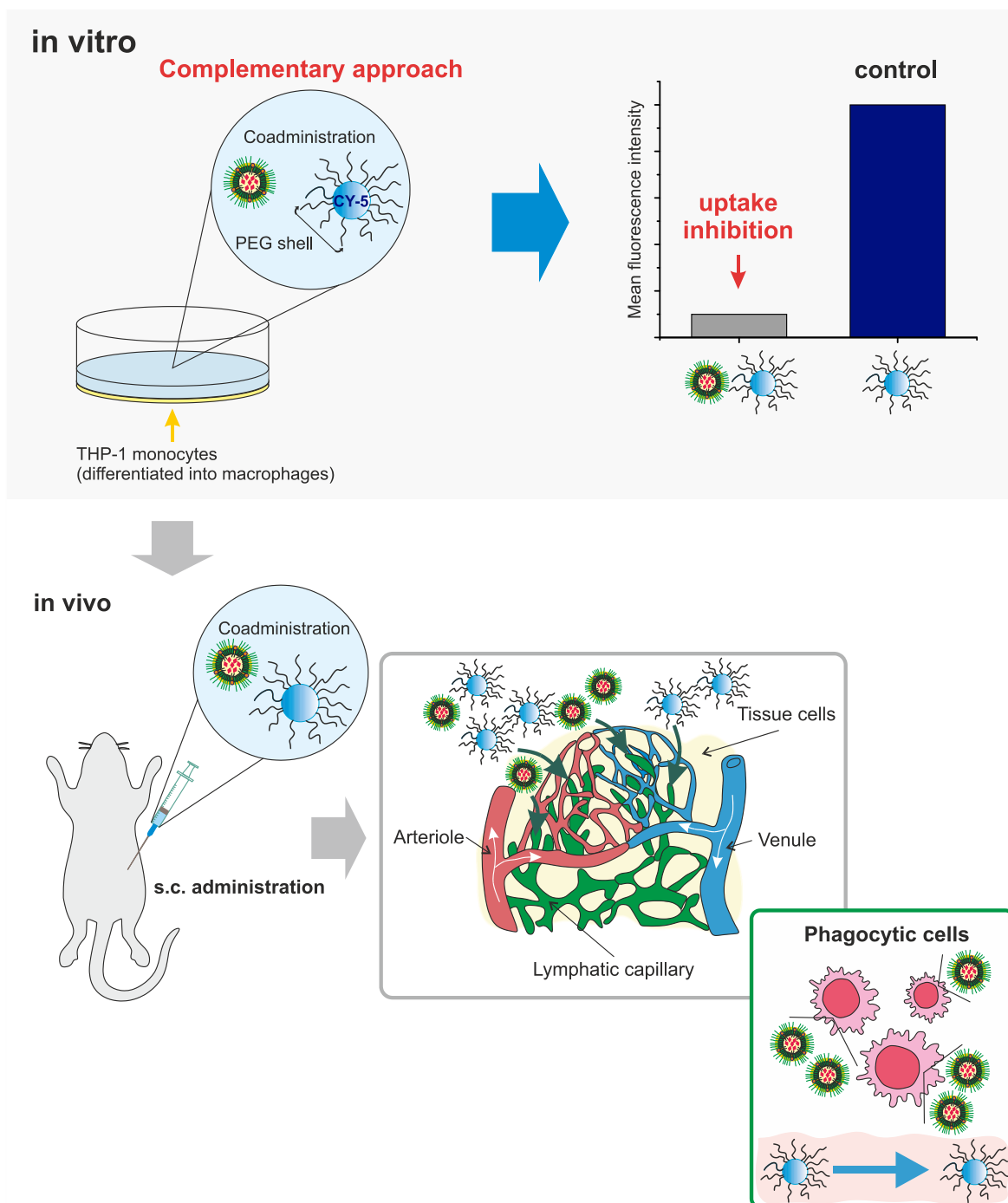
Chapter 6

A Complementary Approach for the
Subcutaneous Administration of Polymer
Nanoparticles - An in vitro Study

Manuscript

Abstract

In the present study, the aim was to achieve systemic availability of polymer nanoparticles at relevant concentrations following subcutaneous administration. This remains a significant challenge, as nanoparticles are quantitatively recognized and sequestered by cells of the mononuclear phagocyte system upon subcutaneous administration. The challenge was tackled by pursuing a complementary approach in which the nanoparticles were protected from excessive opsonization by coating their surface with PEG. Additionally, a coadministration regime with lipid-based decoy nanoparticles was developed, which were loaded with chloroquine, an endocytosis-inhibitor. In vitro, it was successfully demonstrated that preincubating macrophages with chloroquine-loaded decoy particles resulted in a statistically significant uptake inhibition of subsequently administered polymer nanoparticles. The in vitro established coadministration regime thus lays the groundwork for future in vivo studies.



Graphical Abstract

Introduction

The development of biomedical nanoparticles has been driven by the presumptions that they could serve as innovative drug delivery systems, favorably influencing the distribution of drugs towards the target sites and improving their toxicity profiles.^[1] While these approaches show great promise in vitro, the respective in vivo performance of nanoparticles falls short of expectations. A widely cited meta-analysis by Wilhelm et al.^[2] have demonstrated that, on median, only 0.7 % of tumor-targeted nanoparticles successfully reached their targets in vivo. A key limitation affecting delivery efficiency is that, following their systemic administration, 95 % of the nanoparticles are sequestered by cells of the mononuclear phagocyte system (MPS) and subsequently accumulated in off-target tissues, especially the liver.^[3–6] Upon injection, the nanoparticles are quickly identified by the immune system as foreign, pathogen-like material.^[7–9] Consequently, they undergo opsonization and are removed from the systemic circulation by phagocytic cells, e.g. macrophages.^[10,11] The resulting reduced bioavailability of the nanoparticles dramatically affects their targeting efficiency, even following systemic (intravenous) administration. As a result, non-systemic administration regimes have rarely been discovered for nanoparticles, despite their prospective benefits for future applications of nanoparticles as drug products.

Compared to intravenous application, subcutaneous application represents a more convenient administration route, as it allows patients to manage their treatment independently of healthcare professionals.^[12] The inherent challenge of applying nanoparticles subcutaneously, however, is determined by the fact that their size (typically > 10 nm and ≤ 100 nm) prevents their direct uptake from the subcutaneous tissue into the systemic circulation via small blood vessels.^[13–15] Instead, they must enter the blood circulation via the lymphatic system (Figure S1).^[16] In the lymph vessels, the nanoparticles are quantitatively cleared by MPS cells, a process referred to as “lymphatic first-pass transit”.^[16] To discover subcutaneous administration regimes for nanoparticles, consequently, strategies must be developed to circumvent their premature clearance by the MPS within the lymph vessels.^[17]

Hitherto, several engineering-driven approaches to adapt nanoparticles to the organism and shield them from immune system detection have been published.^[18] Systematic studies were conducted to optimize nanoparticles in terms of their size and surface charge.^[19–21] Furthermore, the nanoparticles were grafted with polyethylene glycol (PEG)^[22] or zwitterionic polymers,^[23] or pre-coated with selected proteins^[24,25] to reduce the nanoparticle-induced complement activation and their opsonization in vivo. More advanced strategies include the surface coating of nanoparticles with cell membranes^[26] and the employment of living cells as transport vesicles, referred to as “cell hitchhiking”.^[27] Although all the aforementioned approaches significantly extended the circulation half-life of nanoparticles after systemic administration, they did not achieve a breakthrough success.^[28] Therefore, a distinct emphasis on transiently disabling the MPS to delay or reduce the recognition of nanoparticles by the immune system has recently emerged in research.^[4,29] For this purpose, coadministration regimes were developed to temporarily saturate the uptake capacity of

the macrophages with large doses of non-toxic decoy agents, e.g. nanomaterials.^[30–32] This enables the effector nanoparticles (ENPs) to reach their target upon subsequent application. Additionally, endocytosis inhibitors were tested to specifically suppress the phagocytosis of ENPs in MPS cells.^[3,33] Chloroquine, a FDA-approved antimalarial medication, is one of the lead compounds investigated as MPS modulating drug.^[28,34] The drug reduces the expression of phosphatidylinositol binding clathrin assembly protein (PICALM) and thus interferes with the function of clathrin-mediated endocytosis. It also inhibits the lysosomal pathways of phagocytic cells.^[34] Wolfram et al.^[34] have shown that the uptake of intravenously applied nanoparticles by macrophages as well as their liver and spleen accumulation were significantly reduced, after preconditioning the organism with chloroquine. Another approach being discussed is a drug induced (transient) depletion of macrophages.^[8,35,36] However, since the cells of the MPS play a crucial role in the immune defense, their depletion could negatively impact the organism's immune status and must be critically evaluated.^[37]

Complementary approaches of nanoparticle engineering, e.g. PEGylation, in combination with temporarily modulating of the immune system are considered to be most promising. They are already successfully used to enhance the targeting efficiency of nanoparticles after intravenous administration.^[34] Therefore, the aim of the present study was to develop an analogous complementary approach for a subcutaneous administration regime. CY-5-labeled polymer core-shell nanoparticles composed of poly(D,L-lactide-co-glycolide) (PLGA) covalently coupled to cyanine-5-amine as core material and poly(ethylene glycol)-b-poly(D,L-lactide) (PLA-PEG) copolymer as an adhering shell were employed as model ENPs. They have previously been evaluated to be successful drug delivery systems.^[38,39] To make them accessible for subcutaneous administration, several decoy agents were tested in vitro (Figure 1). Therefore, THP-1 monocytes were employed as target cell line and differentiated into macrophages. Lipid nanocapsules (LNCs) and unstained polymer nanoparticles were evaluated as decoy agents in coadministration regimes with the ENPs via flow cytometry experiments. The mean fluorescence intensity associated with the macrophages was used as an indicator for the uptake of the CY-5-labeled ENPs. Furthermore, chloroquine, as a well-characterized lead compound, was applied in various coadministration regimes. To locally disable the immune system within the lymph and thus mitigate nanoparticle clearance, the coadministered chloroquine must enter the systemic circulation via the lymph vessels. Therefore, the endocytosis inhibitor was encapsulated into LNCs. In a final step, the drug-loaded LNCs were modified by incorporating 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (DOPS), an “eat-me sequence” for macrophages,^[40–42] into their structure. The effect of these engineered LNCs was critically evaluated. The present study was conducted in vitro but designed with the long-term goal to transfer the coadministration regime to an in vivo setting.

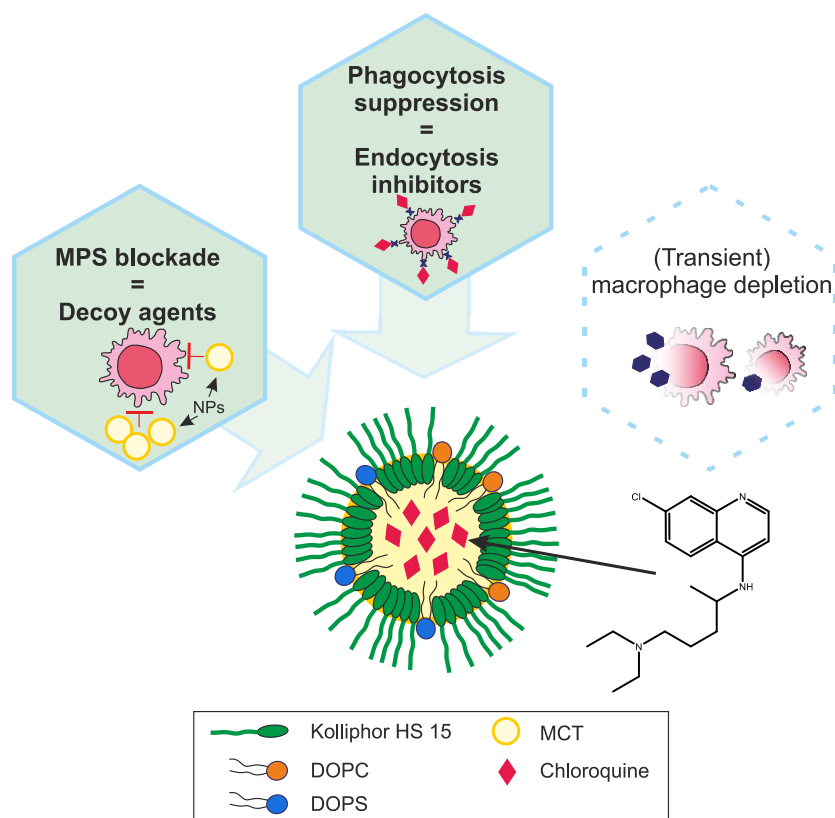


Figure 1 Schematic depiction of strategies to evade clearance by the MPS.^[18] The use of nanoparticulate materials to block the MPS through overload is shown, as well as the application of endocytosis inhibitors to suppress the phagocytosis of effector nanoparticles. Both strategies result in a transient modulation of the immune system. As a third strategy, the use of active components for macrophage depletion is presented, which can sustainably affect the immune system. In the present study chloroquine was used as a endocytosis inhibitor encapsulated into LNCs (100 % DOPC) or engineered LNCs (100 % DOPS).

Results

Characterization of the Model Effector Nanoparticles

The nanoparticles chosen as a model for ENPs in this study were designed as PLGA/PLA-PEG core-shell nanoparticles, already well established in our group^[43,44] and characterized in Chapters 3 - 5.^[45,46] The nanoparticles were obtained by nanoprecipitation, with the hydrophobic PLGA and PLA aggregating in the nanoparticle core.^[47,48] This results in the hydrophilic PEG chains extending into a brush-like corona from the core. The hydrodynamic diameter of the ENPs was determined to be 81.78 ± 2.92 nm (mean $\pm$ SD from three independent batches) using DLS measurements and to be 87.13 ± 3.81 nm (mean $\pm$ SD from three independent batches) using NTA measurements. The morphology of the nanoparticles and considerations on the PEG conformation are described in Chapter 3^[45] and Chapter 4. Carboxy-terminated PEG chains were chosen for engineering the nanoparticle shell. The negative surface charge, indicated by their zeta potential of -29.1 ± 20.0 mV (mean $\pm$ SD from three independent batches), increases the stability of the nanoparticles in aqueous media and prevents their aggregation. PLGA was covalently labeled with cyanine-5-amine to make the nanoparticles accessible for detection in flow cytometry experiments. In preparation for future in vivo studies, it has been successfully demonstrated (Chapter 5)^[46] that the polymer ENPs can be furthermore radioactively labeled with ^{99m}Tc via direct incorporation of the radionuclide into the nanoparticle structure.

Nanoparticles as Decoy Agents

Unmodified LNCs and unstained polymer core-shell nanoparticles (COOH-NPs), which were composed as the ENPs of PLGA/PLA-PEG, were evaluated as decoy agents to mitigate the uptake of ENPs into macrophages differentiated from THP-1 monocytes. The tested decoy particles were intended to occupy the macrophages and thus to temporarily saturate their uptake capacity.^[4,18] The LNCs were produced via phase inversion cycles^[49] and their hydrodynamic diameter was determined to be 41.65 ± 0.72 nm with a PDI of 0.036 ± 0.009 (mean $\pm$ SD from three independent batches) using DLS measurements. The hydrodynamic diameter of the unstained COOH-NPs was determined to be 83.2 ± 0.8 nm (mean $\pm$ SD) using NTA measurements. The efficiency of these two particle types to modulate the ENPs' uptake into macrophages was assessed by flow cytometry experiments. Therefore, the macrophages were preincubated with unstained decoy nanoparticles according to application regimes (Figure 2A, Figure S2) aligned with previously published protocols in the literature.^[17] After the preincubation period, the CY-5-labeled ENPs were added at concentrations of 0.1 nM and 0.01 nM for 0.5 h without removal of the decoy nanoparticles. The subsequently measured mean fluorescence intensity (APC-A geo. mean) was assessed as an indicator for the uptake of the ENPs into macrophages. LNC concentrations ranging from 3 nM to 0.3 nM, along with COOH-NPs concentrations in the range of 1 nM and 0.1 nM were evaluated. The measurement of cell-associated fluorescence indicated that the uptake of ENPs into macrophages remained

unchanged or increased following preincubation with LNCs or COOH-NPs compared to the control experiment of macrophages without preincubation (Figure 2B, Figure S2). Toxicity studies of LNCs and COOH-NPs were conducted on macrophages. The obtained results revealed a reduced cell viability following the incubation with higher LNC concentrations for 24 h. The other tested LNC concentrations and COOH-NPs exhibited toxicity levels below the ISO cytotoxicity threshold (Figure S3A and B).

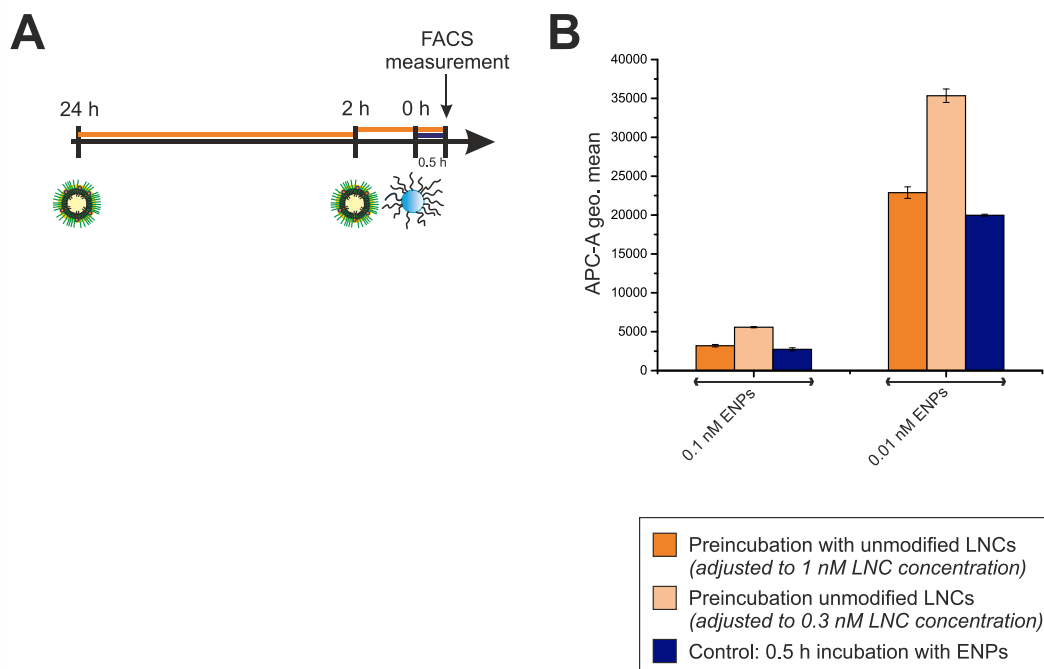


Figure 2 Evaluation of unmodified LNCs as decoy agents. (A) Visualization of the administration regime: The macrophages were pretreated with either a dual application of unmodified LNCs for 24 h and 2 h or a single application for 2 h before the CY-5-labeled ENPs were added for further 0.5 h. (B) The mean fluorescence intensity (APC-A geo. mean) was quantified using flow cytometry analysis. Unmodified LNCs were applied at concentrations of 1 nM or 0.3 nM and the ENPs at concentrations of 0.1 nM and 0.01 nM. Macrophages, which were treated exclusively with ENPs, without prior incubation with unmodified LNCs, served as control. Results represented as mean $\pm$ SD ($n = 3$).

Chloroquine Diphosphate for Transient Phagocytosis Inhibition

The effect of chloroquine diphosphate, reported as an inhibitor of clathrin-mediated endocytosis,^[28,34] on the uptake of ENPs into macrophages was assessed using flow cytometry experiments. Its efficiency was analyzed within a concentration range of 20 μ M to 400 μ M. This range was documented in the literature to be potent for nanoparticle uptake inhibition,^[34] across incubation intervals of up to 24 h. The preincubation with chloroquine diphosphate resulted in a distinct inhibition of ENP uptake relative to the control, with increasing uptake inhibition at higher chloroquine diphosphate concentrations (200 μ M, 400 μ M) and extended preincubation durations (24 h) (Figure S4A). Lower chloroquine diphosphate concentrations showed uptake inhibition only after a

preincubation period of 24 h, as compared to the control. In subsequent experiments, it was assessed whether the uptake inhibition effect persisted when the macrophages were incubated with ENPs for longer than 0.5 h. It was demonstrated that chloroquine diphosphate reliably induced uptake inhibition for at least 2 h (Figure S4B). The effect was thoroughly evaluated across a concentration range of ENPs from 1 nM to 1 pM by pretreating macrophages with chloroquine diphosphate at concentrations of 100 μ M and 200 μ M. Compared to the controls, the preincubated macrophages exhibited a significantly reduced uptake efficiency for ENPs across the entire concentration range over 4 h (Figure 3A and B). Toxicity studies of the administration regime of chloroquine diphosphate and ENPs indicated toxicity levels below the ISO cytotoxicity threshold for chloroquine diphosphate at a concentration of 100 μ M over 4 h, while a concentration of 200 μ M resulted in a decreased cell viability (Figure S3C).

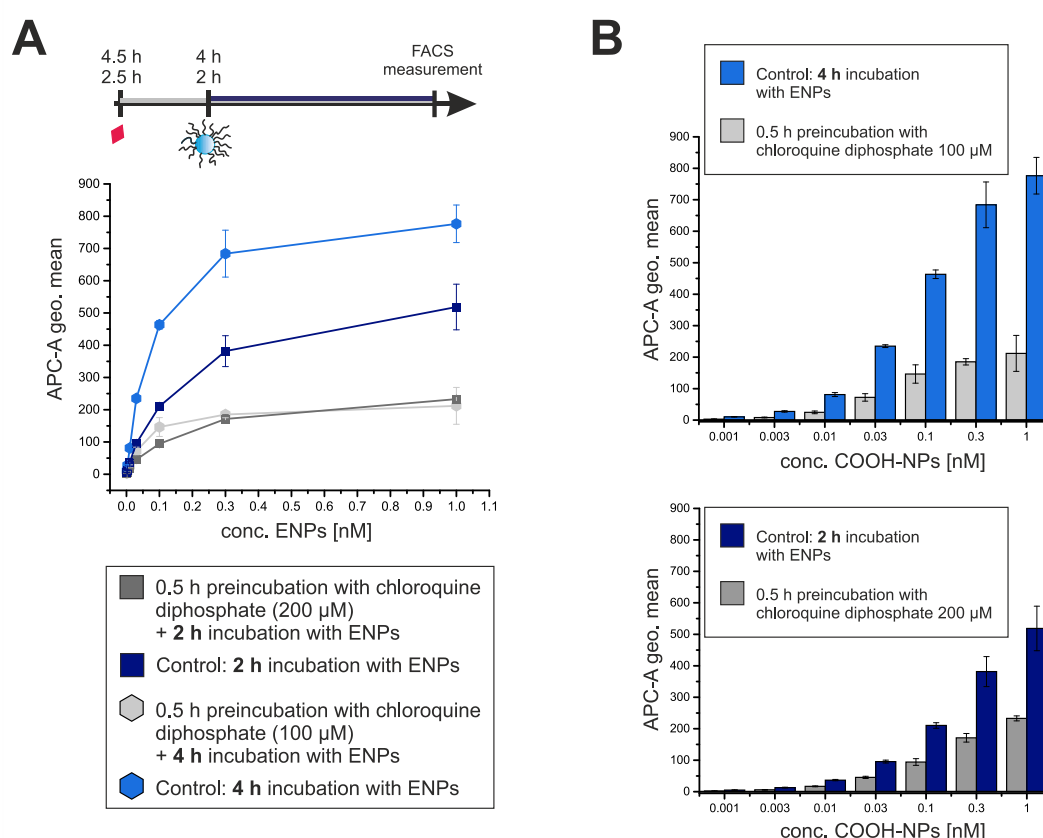


Figure 3 Evaluation of chloroquine diphosphate as an endocytosis inhibitor. The mean fluorescence intensity (APC-A geo. mean) was assessed as an indicator for the uptake of CY-5-labeled ENPs into macrophages. Results represented as mean $\pm$ SD ($n = 3$). Macrophages, which were treated exclusively with ENPs, without prior incubation with chloroquine diphosphate, served as control (indicated in blue). (A) Visualization of the administration regime: Macrophages were preincubated with chloroquine diphosphate for 0.5 h before the ENPs were added for further 4 h or 2 h. Chloroquine diphosphate was applied at concentrations of 200 μ M or 100 μ M. The ENPs were applied in serial dilutions (1 nM to 1 pM). Controls are indicated in blue. (B) Evaluation of concentration-dependent effects on the uptake-inhibition of ENPs by chloroquine diphosphate.

Chloroquine-Loaded LNCs as Decoy Agents

To make chloroquine diphosphate deliverable for the inhibition of phagocytotic cells in the lymph, the active compound was encapsulated in unmodified LNCs, which had been previously validated as robust drug delivery systems.^[49,50] Therefore, chloroquine diphosphate was converted into its free base via liquid-liquid extraction. The purity and identity of the chloroquine free base was verified against a commercially obtained reference using HPLC analysis (Figure S5). Both, the extracted chloroquine free base and the reference showed a constant retention time of 2.9 min. In anticipation of future in vivo studies on rats, the encapsulated chloroquine dosage was optimized to align with the required in vivo dose for immunomodulation, ensuring compliance with standard administration volumes^[51] and toxicity thresholds.^[3] For the planned application volume of 250 μL , the concentration of encapsulated chloroquine had to be adjusted to 6 mg mL^{-1} per batch. (*The reader is referred to the Supporting Information.*) The encapsulation of chloroquine free base into unmodified LNCs resulted in an encapsulation efficiency (*EE*) of $73.9 \pm 7.5\%$ (mean $\pm$ SD; $N = 2$; $n = 3$) determined by HPLC analysis. The efficiency of chloroquine-loaded unmodified LNCs on the uptake inhibition of ENPs into macrophages was assessed in flow cytometry experiments. Therefore, the macrophages were preincubated with the LNCs according to the already established administration regime (Figure 4A). The applied LNC concentrations were adjusted to obtain chloroquine concentrations of 100 μM , 200 μM and 300 μM . The corresponding LNC particle concentrations were ≤ 0.7 nM. The ENPs were applied at concentrations ranging from 0.1 nM to 0.01 nM and the mean fluorescence intensity (APC-A geo. mean) was assessed as an indicator for their uptake (Figure 4B). The efficiency of chloroquine-loaded LNCs on the uptake inhibition was examined using one-way ANOVA with subsequent Bonferroni corrected post hoc paired t-test, as the Levene test indicated variance homogeneity. Preincubation with chloroquine-loaded unmodified LNCs resulted in a statistically significant reduction of the uptake of ENPs over 4 h compared to macrophages that were not preincubated. As an additional control experiment, macrophages were preincubated with unloaded LNCs, which, as previously described, resulted in an either unchanged or increased uptake of ENPs compared to macrophages, which were not preincubated.

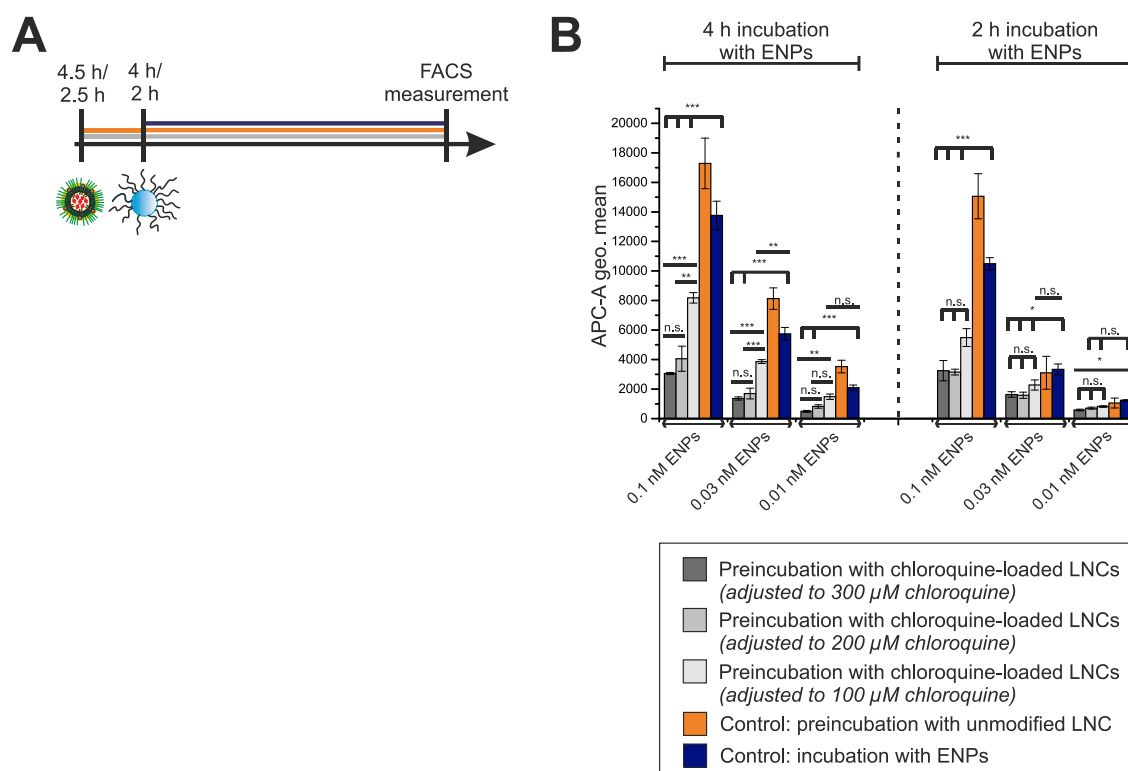


Figure 4 Evaluation of chloroquine-loaded unmodified LNCs as decoy agents. (A) Visualization of the administration regime: The macrophages were preincubated with chloroquine-loaded unmodified LNCs for 0.5 h before subsequent incubation with CY-5-labeled ENPs for 4 h or 2 h. (B) The mean fluorescence intensity (APC-A geo. mean) was assessed as an indicator for the uptake of ENPs into macrophages. Results represented as mean $\pm$ SD ($n = 3$). Macrophages, which were treated exclusively with ENPs, without prior incubation with chloroquine-loaded LNCs, and macrophages, which were pretreated with unloaded LNCs before incubation with ENPs, served as control. The statistical significance between the means was evaluated using one-way ANOVA with subsequent Bonferroni corrected post hoc paired t-test, as the Levene test indicated variance homogeneity. Levels of statistical significance are indicated as n.s. – not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

The Effect of Engineered LNCs

1,2-Dioleoyl-*sn*-glycero-3-phosphoserine (DOPS) was incorporated as a targeting structure into the decoy LNCs (= engineered LNCs) to provide an additional uptake stimulus for macrophages.^[42] Therefore, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was partially replaced by DOPS during the production of the LNCs (Figure 5A). The engineered LNCs, with DOPS contents ranging from 25 % to 100 % (m/m), exhibited hydrodynamic diameters between 40.08 ± 0.01 nm and 42.94 ± 0.45 nm along with PDIs ranging from 0.03 ± 0.01 to 0.07 ± 0.02 (mean $\pm$ SD from three independent batches) (Figure 5B). To assess the uptake-enhancing effect of DOPS, the LNCs were stained with DiO and applied to macrophages for 0.5 h and 1 h. Unmodified LNCs (0 % DOPS) served as control. The mean fluorescence intensity (APC-A geo. mean) was measured via flow cytometry experiments and assessed as an indicator for the uptake of engineered LNCs into macrophages (Figure 5C). In both experimental settings, a statistically significant difference in the uptake of LNCs into macrophages was only observed for DOPS contents of at least 75 %.

Subsequently, further investigations were conducted using LNCs, in which DOPC was substituted quantitatively with DOPS (100 %-DOPS-LNCs).

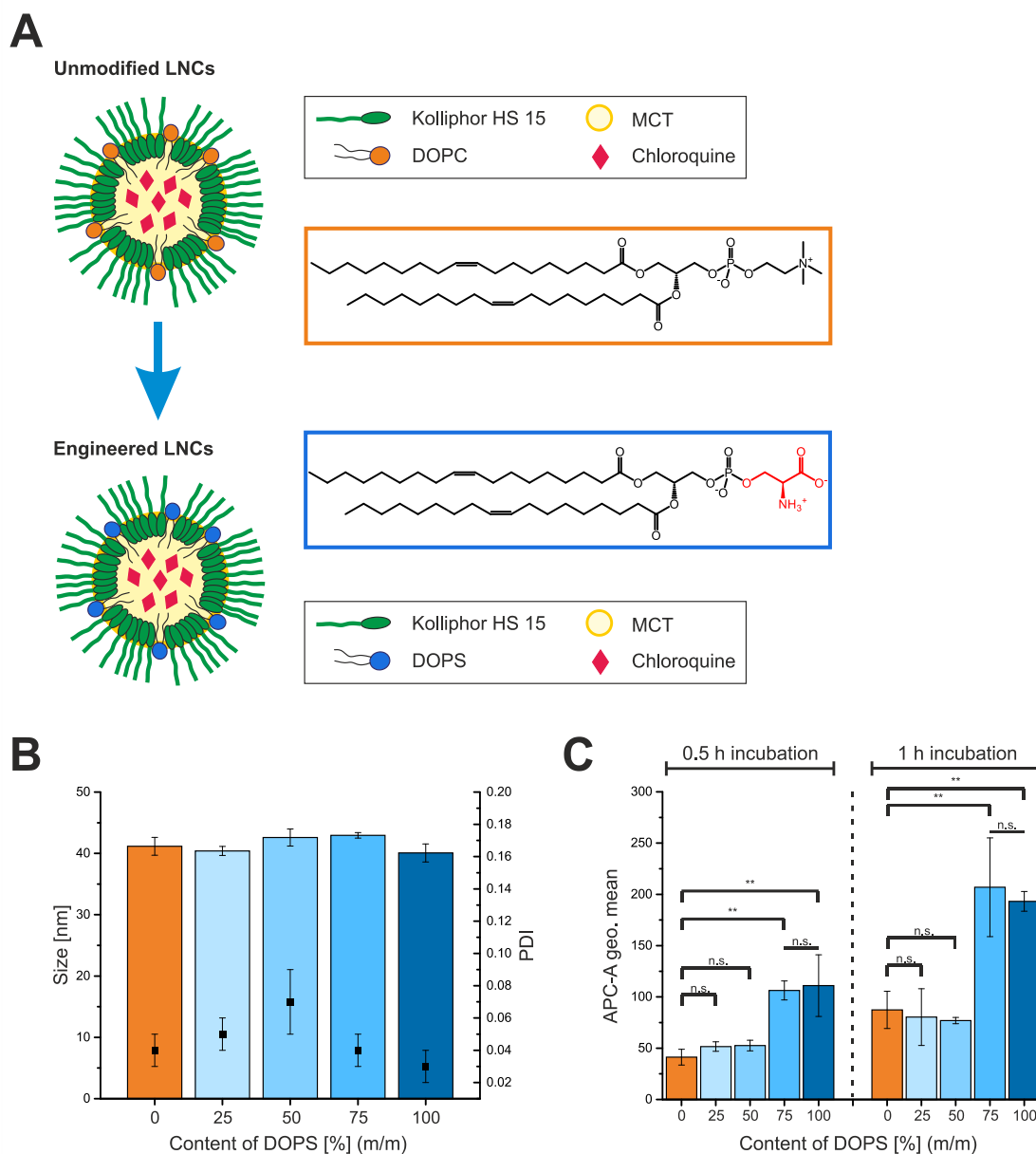


Figure 5 Engineering LNCs with DOPS as an “eat-me” signal to trigger their phagocytosis. (A) Schematic illustration of the chemical composition of unmodified and engineered LNCs and the chemical structures of DOPC and DOPS. (B) Size (dh) [nm] and PDI of LNCs with varying contents of DOPS [%] (m/m). DLS data represented as mean $\pm$ SD (N = 3; n = 3). (C) Investigation of the LNC uptake into macrophages as a function of their DOPS content [%] (m/m). Results represented as mean $\pm$ SD (n = 3). The statistical significance between the means was evaluated using one-way ANOVA with subsequent Bonferroni corrected post hoc paired t-test, as the Levene test indicated variance homogeneity. Levels of statistical significance are indicated as n.s. – not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Chloroquine free base was encapsulated into 100 %-DOPS-LNCs. Using HPLC analysis, the *EE* was determined to be 88.0 ± 27.0 % (mean $\pm$ SD; N = 2; n = 3). The chloroquine-loaded 100 %-DOPS-LNCs differed statistically significantly in their size from unmodified chloroquine-loaded LNCs

(Two Sample t-test: $p\text{-value} = 6.424 \cdot 10^{-7}$) (Figure 6A). To compare their effect on the uptake inhibition of ENPs, the previously described experimental setting and administration regime for flow cytometry experiments was employed (Figure 6B). The applied concentrations of both LNC types were adjusted to obtain chloroquine concentrations of 200 μM . The corresponding LNC particle concentrations were $\leq 0.7 \text{ nM}$. Macrophages, which were preincubated with 200 μM chloroquine diphosphate were set as an additional control. Preincubation with chloroquine-loaded 100 %-DOPS-LNCs and chloroquine-loaded unmodified LNCs resulted in a statistically significant reduction of ENP uptake over 4 h compared to macrophages that were not preincubated with chloroquine-loaded LNCs (Figure 6C). Compared to macrophages which were preincubated with chloroquine diphosphate, the difference in ENP uptake over 4 h was statistically weakly significant or not significant. Furthermore, the analysis of the flow cytometry experiments revealed that preincubation with unloaded, drug-free 100 %-DOPS-LNCs compared to unloaded unmodified LNCs, did not reduce the uptake of ENPs into macrophages but instead resulted in a statistically significant increase.

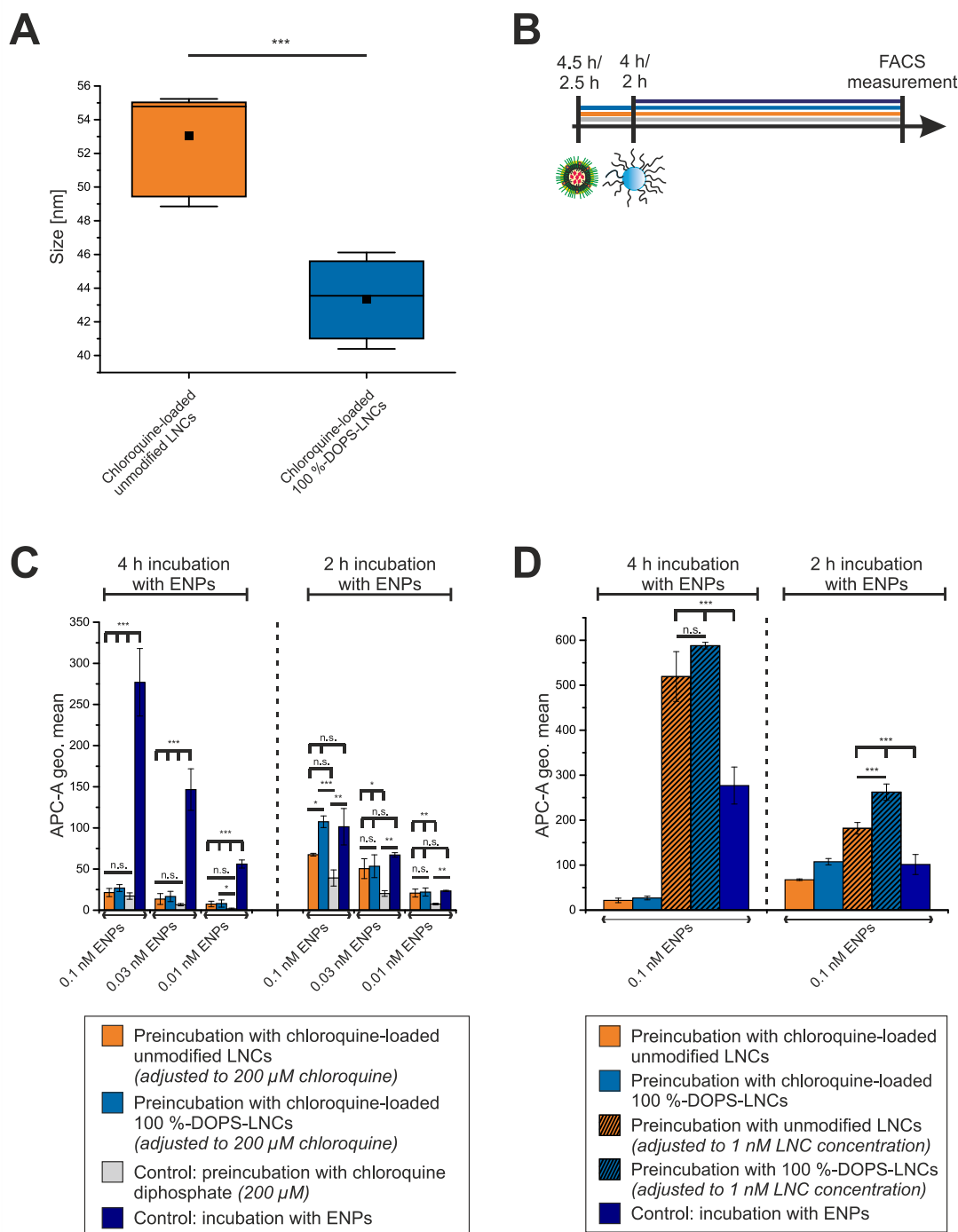


Figure 6 Evaluation of engineered chloroquine-loaded LNCs as decoy agents. (A) Size (dh) [nm] of chloroquine-loaded unmodified LNCs and chloroquine-loaded 100 %-DOPS-LNCs. DLS data represented as mean $\pm$ SD (from three independent batches). Derived means were compared via Two Sample t-test (p -value = 6.424×10^{-7}). Levels of statistical significance are indicated as n.s. – not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. (B) Visualization of the administration regime: The macrophages were preincubated with chloroquine-loaded unmodified LNCs and chloroquine-loaded 100 %-DOPS-LNCs for 0.5 h before subsequent incubation with ENPs for 4 h or 2 h. (C) The mean fluorescence intensity (APC-A geo. mean) was assessed as an indicator for the uptake of ENPs into macrophages. Macrophages, which were treated exclusively with ENPs, without prior incubation with decoy agents, served as a control. Results represented as mean $\pm$ SD ($n = 3$). The statistical significance between the means was evaluated using one-way ANOVA with subsequent Bonferroni corrected post hoc paired t-test, as the Levene test indicated variance homogeneity. Levels of statistical significance are indicated as n.s. – not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. (D) Comparison of the uptake of ENPs after preincubation with chloroquine-loaded unmodified LNCs, chloroquine-loaded 100 %-

DOPS-LNCs, unmodified LNCs and 100 %-DOPS-LNCs. Results represented as mean $\pm$ SD (n = 3). The statistical significance between the means was evaluated using one-way ANOVA with subsequent Bonferroni corrected post hoc paired t-test, as the Levene test indicated variance homogeneity. Levels of statistical significance are indicated as n.s. – not significant, *p $\leq$ 0.05, **p $\leq$ 0.01, ***p $\leq$ 0.001.

Discussion and Conclusion

In this study, a coadministration regime was developed *in vitro* with the aim of ensuring the systemic availability of nanoparticles after subcutaneous administration. This work serves as a preparatory step for future *in vivo* studies. A complementary approach was implemented to reduce the clearance of ENPs by phagocytotic immune cells in the lymph. The ENPs were protected against excessive opsonization through PEGylation of their surface.^[44,52,53] Additionally, their uptake into MPS cells was mitigated by the coadministration of an endocytosis inhibitor, chloroquine. Chloroquine was encapsulated in LNCs and interferes with the clathrin-mediated endocytosis of nanoparticles.^[34] *In vitro*, it was successfully demonstrated that a preincubation with chloroquine-loaded LNCs significantly reduced the uptake of ENPs by macrophages compared to the control group. Due to its molecular weight (< 1 kD), unencapsulated chloroquine would not be quantitatively absorbed via the lymph vessels after subcutaneous administration. Instead, it would enter the systemic circulation through small blood capillaries.^[54] LNCs were chosen as carrier system to promote the chloroquine uptake via the lymph, as they showed excellent encapsulation efficiency for lipophilic drugs as previously published by our group.^[49,50] Moreover, as particulate systems, they exhibit a high potential for interaction with MPS cells.^[4,18] Several studies have already demonstrated that the administration of high doses of non-toxic, unloaded (drug-free) nanoparticles as decoy agents leads to saturation of the uptake capacity of MPS cells, consequently resulting in a reduced uptake of subsequently administered ENPs.^[4,18] However, saturation effects could not be proven after preincubation with either lipid-based or polymer decoy particles in the present study. This could potentially be attributed to the decoy nanoparticle concentrations employed. Ouyang et al.^[55] demonstrated that the threshold for the uptake capacity of Kupffer cells, which are liver specific macrophages, was reached at 1 trillion (gold) nanoparticles in mice. Once this threshold was exceeded, the clearance of systemically administered nanoparticles significantly decreased. However, this threshold did not represent the total capacity of the cells but a kinetic limit. *In vitro*, the uptake stagnated at 100,000 phagocytosed nanoparticles per cell over 24 h (applied nanoparticle concentration: approximately $1.0 \cdot 10^{12}$ particles mL⁻¹). In the present study, the administered decoy nanoparticle concentrations were lower by at least a factor of 1000 due to toxicity considerations. The toxicity of high doses of unloaded decoy particles has already been critically evaluated as a health risk in the literature.^[56,57] The encapsulation of chloroquine made the decoy LNCs effective even at moderate, *in vitro* non-toxic concentrations. Chloroquine possesses an acceptable toxicity profile and has already been utilized as an adjuvant therapy in combination with chemotherapy for extended treatment durations.^[34,58] The endocytosis-inhibitory effect on cells is transient, and they can fully regenerate after the removal of chloroquine. This is highly relevant for the future administration *in vivo*, as a persistent impairment of the immune system would pose significant health risks.^[37]

The incorporation of DOPS, an “eat-me” signal presented by apoptotic cells^[40] and utilized by certain viruses for cell targeting,^[41,59] into the envelope of the decoy LNCs did not enhance their efficiency of uptake-inhibition for ENPs. Although DOPS has already been successfully employed as a ligand-

like structure in particulate systems for targeting macrophages,^[42] its implementation in decoy particles could even prove disadvantageous: DOPS enhances the phagocytosis activity of macrophages and, thus, also for the uptake of ENPs. Instead, the incorporation of “don’t-eat-me” sequences appears to be a promising approach, as reported by Tang and coworkers.^[60] They implemented a CD47-derived peptide ligand into the envelop of decoy particles, which blocked the surface of the macrophages by specific ligand-receptor interaction for the uptake of ENPs. Consequently, this system was not based on the intracellular release of a MPS modulator from the decoy particles.

Various coadministration regimes for (drug-loaded) decoy nanoparticles and ENPs have been published. So far, these approaches have been quantitatively developed for systemic (intravenous) administration with the aim of modulating the target/off-target distribution of the ENPs.^[57,61,62] To the best of our knowledge there is only one other study in which a coadministration regime was utilized to render ENPs systemically available after subcutaneous administration.^[17] Stack et al.^[17] successfully employed lantrunculin A-loaded micelles as decoy particles for subsequent subcutaneous ENP administration. They demonstrated a reversible and non-toxic MPS inhibition, while achieving ENP serum levels after subcutaneous coadministration comparable to those observed after intravenous application. Lantrunculin A, a micropinocytosis inhibitor, has so far not been approved as a drug substance. Both the decoy particles and the ENPs were composed of the same polymer, poly(ethylene glycol)-*block*-poly(propylene sulfide) (PEG-*b*-PPS). In this study, we differentiate our approach by utilizing two distinct particle types, lipid-based decoy particles and polymer ENPs. The concept of coadministration is consequently applicable to mixed-material systems, and our study provides an initial indication of the general transferability of this concept between different nanoparticle types. It is therefore conceivable that an established decoy nanoparticle system could be combinable with various ENPs, which are optimized for their targets.

Aside from this, nanoparticles have thus far only been administered subcutaneously as depots for the sustained release of their cargo.^[54,63–65] The sizes of the nanoparticles (> 100 nm) were chosen to prevent their entry into the lymphatic system, ensuring the release of the loaded drugs into the subcutaneous tissue.^[54] Following subcutaneous administration, drug substances first pass the interstitium to reach the lymphatic vessels and blood capillaries.^[54] Their encapsulation within nanoparticles further amplifies this naturally occurring retardation by the influence of the release kinetics from the nanoparticle depot. If the entire nanoparticle successfully reaches the systemic circulation after subcutaneous administration, a retardation can consequently be expected compared to its intravenous administration. This could have beneficial effects on the targeting efficiency of the nanoparticles as drug delivery systems^[66] as well as on their toxicity profiles. It could prevent an overload of the liver, which typically occurs after systemic administration.^[56]

Taken together, the proposed coadministration regime potentially represents a novel application route for nanoparticles, enabling their sustained release into the systemic circulation. The subcutaneous application of nanoparticles would therefore no longer be limited to its use as drug-releasing depots. Furthermore, the coadministration regime constitutes a transferable approach

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applicable to various ENPs. However, assessing the functionality and efficacy of the coadministration regime in vivo remains a critical factor.

Material and Methods

Materials

All materials and reagents were purchased from Sigma Aldrich (Taufkirchen, Germany or St. Louis, USA) unless otherwise stated. Dulbecco's phosphate-buffered saline (DPBS) was acquired from Gibco™ (Life Technologies, Paisly, UK). The ultrapure water used for the experiments was treated with a Milli-Q EQ 7000 system (Merck, Darmstadt, Germany) equipped with Milli-Q Biopak filter (Merck, Darmstadt, Germany) and freshly taken every day. It is referred to as Milli-Q water in the following.

Cell Culture

Human acute monocytic leukemia cells (THP-1 monocytes) were cultured in RPMI 1640 cell culture medium (gibco, RPMI Medium 1640 (1X), [-]-L-Glutamine, Thermofisher, Paisley, UK) containing 10 % fetal bovine serum (FBS) (South America Origin, Pan-Biotech, Aidenbach, Germany, Lot-No.: P201004) and supplemented with MEM non-essential amino acids, MEM vitamins, sodium pyruvate and glutamine (gibco, Life Technologies Corporation, Grand Island, USA). For the differentiation into macrophages, 100 nM phorbol-12-myristate-13-acetate (PMA) (ThermoScientific Fisher, Schwerte, Germany) was added to the cell culture medium 48 h before the experiment. The cells were cultured in a water saturated, carbon dioxide enriched (5 % CO₂) atmosphere at 37 °C.

Nanoparticle Preparation

Core-shell nanoparticles were obtained by nanoprecipitation.^[46,67] For this purpose, the synthesized PLA_{10K}-PEG_{5K}-COOH block-copolymer was adjusted together with PLGA or CY-5 labeled PLGA (CY-5-PLGA) in acetonitrile (ACN) to a concentration of 10 mg mL⁻¹. The ratio of the PLGA or CY-5-PLGA core to block-copolymer shell was 3:7 (m/m). The organic phase was added dropwise into the 10-fold excess of stirring (930 rpm) aqueous phase (10 % DPBS (v/v) in Milli-Q water) and stirred for 3 h to remove the ACN. Subsequently, the volume was concentrated by centrifugation at 3000 g for 15 min using Microseps Advance 30K centrifugal filters (molecular weight cut-off 30 kDa, Pall Corporation, New York, USA).

Lipid Nanocapsules (LNC) Preparation

LNCs were manufactured as previously published by our group.^[68] For unmodified LNCs, 295.8 mg Kolliphor® HS15 solution 40% (m/m) in Milli-Q water, 18.3 mg (13.3-23.3 mg) 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC, LIPOID PC 18:1/18:1, kindly provided by LIPOID GmbH, Ludwigshafen, Germany), 138.3 mg Miglyol® (Miglyol® 812, Ph.Eur.8.0, Caesar & Loretz GmbH, Hilden, Germany) and 222.6 mg aqueous NaCl solution (50 mg mL⁻¹) were mixed and subjected to

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three heating (to 90 °C)-cooling (to 60 °C) cycles. In the last cooling cycle 850 µL aqueous NaCl solution were added at the phase inversion temperature (approx. 76 °C) to form stable LNCs. The LNC emulsion was stirred for further 5 min and then sterile filtered through a RC-filter (0.22 µm) to remove excess lipid. The LNC emulsion was stored at 2-8 °C protected from light. For engineered LNCs, DOPC was replaced proportionately by 1,2-dioleoyl-*sn*-glycero-3-phosphoserine, sodium salt (DOPS-Na, LIPOID PS 18:1/18:1, kindly provided by LIPOID GmbH, Ludwigshafen, Germany) and the manufacturing process was completed as described above. For selected flow cytometry experiments, the LNCs were labeled with DiO (DiOC18(3), Invitrogen, Thermo Fisher Scientific, OR, USA). In this case, 0.5 mg DiO were added to the mixture before it underwent the heating-cooling cycles.

	Amount of DOPC [%]	Amount of DOPS [%]
Unmodified LNCs	100	0
Engineered LNCs	75	25
	50	50
	25	75
	0	100

Nanoparticle and LNC Characterization

Nanoparticle characterization:

Dynamic light scattering (DLS)-based measurements (particle size distribution, polydispersity index) were carried out with a Malvern Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany) equipped with a λ 633 nm He-Ne laser operating at an angle of 173°. The software used for data collecting and processing was Zetasizer software version 7.12 (Malvern Instruments, UK). To determine the size distribution, the concentrated samples were diluted 1:20 with 10 % DPBS in Milli-Q water (10 % DPBS (v/v)). 90 µL sample volume were measured in micro-UV-cuvettes (Carl Roth, Karlsruhe, Germany) at a controlled temperature of 25 °C (120 sec equilibration time). Three repetitions with 11 runs of 10 sec duration were performed for each sample. The data was analyzed with the software-integrated analysis model for “general purposes (normal resolution)”.

Furthermore, the average size (hydrodynamic diameter, dh) and concentration [particles mL⁻¹] of the nanoparticles were determined using nanoparticle tracking analysis (NanoSight NS300, Malvern, Worcestershire, UK). The device was equipped with an O-Ring top-plate and the data were analyzed with NanoSight NTA 3.4 software (NTA Malvern Panalytical software). Before measurement, all samples were diluted with Milli-Q water to achieve a good particle per frame value (20-100 particles/frame). The samples were applied by manual injection with sterile syringes. Each sample was measured in three cycles of 60 sec duration (screen gain: 1.0; camera level: 16) at RT. The detection threshold was set at 3.

LNC characterization:

Particle size distribution and polydispersity index measurements were carried out with a Malvern Zetasizer Nano ZS as for the polymer nanoparticles. Therefore, the LNC samples were diluted 1:20 with 10 % DPBS in Milli-Q water. 90 μ L sample volume were measured in micro-UV-cuvettes (Carl Roth, Karlsruhe, Germany) at a controlled temperature of 25 °C (120 sec equilibration time). Three repetitions with 11 runs of 10 sec duration were performed for each sample. The data was analyzed with the software-integrated analysis model for “general purposes (normal resolution)”. Furthermore, the average size (hydrodynamic diameter, dh) and concentration [particles mL^{-1}] were determined using nanoparticle tracking analysis as described above. Before measurement, the samples were diluted with Milli-Q water. The samples were applied by manual injection with sterile syringes. Each sample was measured in three cycles of 60 sec duration (screen gain: 1.0; camera level: 16) at RT. The detection threshold was set at 2.

Chloroquine Free Base Synthesis and Characterization

About 1 g of the chloroquine diphosphate salt was placed in the separating funnel, and the free base of the drug was extracted by liquid-liquid extraction with ethyl acetate (50 mL) and sodium hydroxide solution (2 M, 75 mL). The organic layer was separated and shaken again against sodium hydroxide solution (1 M, 75 mL). The solvent was then removed under vacuum for 12 h. Identity and purity of the chloroquine free base were verified by high performance liquid chromatography (HPLC) analyses. HPLC analyses were performed on an Agilent Technologies 1260 Infinity II (Santa Clara, USA). For this, a C18 column (CORTEX T3 2.7 μ m, 3 x 100 mm column) was used, that was heated to 40 °C. Acetonitrile (HPLC grade) [A] and 0.1 % triethylamine in Milli-Q water (pH 3.0 adjusted with phosphoric acid) [B] were employed as the mobile phases for the gradient elution at a flow rate of 1.0 mL min^{-1} , as previously published by Miranda et al.^[69] The following gradient was applied: [A] at 5 % - 0-2 min/ [A] 5 to 40 % - 2-4 min/ [A] at 40 % - 4-5.3 min/ [A] 40 to 5 % - 5.3-7 min/ [A] at 5 % - 7-9 min. Fluorescence detection was performed at an excitation wavelength of λ 325 nm and an emission wavelength of λ 375 nm. Emission spectra were recorded in the peaks (scan range: λ 335 nm to 400 nm, step: 10 nm). 2 μ L sample volume (dissolved in methanol and aqueous phase [B], ratio 1:1) were applied in each run. The extracted chloroquine free base showed a constant retention time of 2.9 min and was compared to chloroquine free base (Reference) purchased from MedChemExpress (Hölzel Diagnostika Handels GmbH, Köln, Germany).

Chloroquine Encapsulation and Quantification via HPLC Analysis

To prepare chloroquine-loaded LNCs, 24 mg chloroquine were dissolved in dichloromethane and added to the mixture of Kolliphor® HS15, Miglyol® and DOPC/DOPS. The dichloromethane was evaporated at 75 °C for 30 min. The aqueous NaCl solution was then added, and the heating-cooling-cycles were initiated as described above. Free non-encapsulated chloroquine was separated by centrifuging the LNC batches twice (2x addition of 1.0 ml Milli-Q water for washing, centrifugation at

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4500 g for 30 min) using Microseps Advance 30 K centrifugal filters (molecular weight cut-off 30 KDa, Pall Corporation, New York, USA). The filtrate was then adjusted to a final volume of 2.0 mL with Milli-Q water. 200 μ L of the LNC emulsion were diluted 10-fold with methanol. The samples were ultrasonicated for 30 min to disrupt the LNC structure and to release the drug, which was quantified by HPLC analyses. Therefore, 100 μ L of the sample were diluted with 100 μ L mobile phase [B] (0.1 % triethylamine in Milli-Q water (pH 3.0 adjusted with phosphoric acid)). The HPLC analyses were performed as described above for the free chloroquine base. However, the gradient was extended to reliably elute the lipids from the HPLC column, which had no effect on the retention time of the chloroquine base. The following gradient was applied: [A] at 5 % - 0-2 min/ [A] 5 to 40 % - 2-4 min/ [A] at 40 % - 4-5.3 min/ [A] 40 to 90 % - 5.3-9 min/ [A] 90 to 5 % - 9-10 min/ [A] at 5 % - 10-15 min. The samples were analyzed in triplicate and the mean drug content [mg mL^{-1}] (mean $\pm$ SD; $n = 3$) was calculated using a previously recorded calibration curve (chloroquine conc.: 0.1 – 0.8 mg mL^{-1}) with the regression coefficient of $R^2 = 0.9989$. The encapsulation efficiency (EE) was calculated as depicted in equation 1, with m_e referring to the mass of encapsulated drug and m_a referring to the mass of initially added drug:

$$EE [\%] = \frac{m_e}{m_a} \cdot 100$$

eq. 1

Flow Cytometry

THP-1 monocytes were seeded into 48-well plates at a density of 120,000 cells/well. The monocytes were differentiated into macrophages by addition of 100 nM PMA to the cell culture medium and incubated for 48 to 72 h (37 °C, 5 % CO_2) before proceeding the flow cytometry experiment. At the day of the experiment, the cells were washed with pre-warmed DPBS before preincubation with the decoy agents (unstained COOH-NPs, conc.: 1 nM, 0.3 nM, 0.1 nM/ unmodified and unstained LNCs, conc.: 3 nM, 1 nM, 0.3 nM/ engineered LNCs, conc.: 1 nM) or the endocytosis inhibitor (aqueous chloroquine dilution (chloroquine diphosphate) conc.: 20 μ M, 100 μ M, 200 μ M, 400 μ M)^[34] or the endocytosis inhibitor containing LNCs (chloroquine containing unmodified or engineered LNCs, adjusted to chloroquine conc. of 100 μ M, 200 μ M, 300 μ M chloroquine) under moderate shaking (50 rpm) at 37 °C for 0.5-24 h. After preincubation, CY-5-labeled ENPs were added to the wells without discarding the previously applied material and incubated under moderate shaking (50 rpm) at 37 °C for 0.5-4 h. To determine concentration-dependent effects, serial dilutions (1 nM to 1 pM, final nanoparticle concentration in the cell culture well) of ENPs were prepared in RPMI 1640 medium containing FBS. For the remaining experiments, the ENP concentrations were adjusted to 0.1 nM, 0.03 nM and 0.01 nM (final nanoparticle concentration in the cell culture well). To determine the effect of LNC engineering with DOPS, the cells were incubated with the DiO-stained LNCs for 30 min or 1 h. For this purpose, the nanoparticle concentration in all batches was assumed to be uniform after production, and the batches were diluted in the same ratio (1:66.7) with RPMI 1640 medium containing FBS.

After the incubation period, the medium was discarded. For harvesting, 300 μL of trypsin (0.25 %) (PAN-Biotech, Aidenbach, Germany) were added and the cells were incubated for 2 min (37 °C, 5 % CO_2). After cell detachment, 500 μL of ice-cold RPMI 1640 were added to quench trypsinization. The samples were transferred to 2.0 ml Eppendorf-Tubes and centrifuged (0.3 g, 7 min, 4 °C). The supernatant was discarded, and the cells were washed twice with ice-cold DPBS under centrifugation. The cell pellets obtained after the last washing step were resuspended in 200 μL ice-cold DPBs and kept on ice until measurement. Flow cytometry analyses were performed on a FACS Canto II (Becton Dickinson, Franklin Lakes, USA). The fluorescence of the CY-5-labelled NPs was excited at a wavelength of λ 633 nm and the emission was recorded using a λ 661/16 nm bandpass filter. The fluorescence of the DiO-stained LNCs was excited at a wavelength of λ 488 nm and the emission was recorded using a λ 530/30 nm bandpass filter. The data was analyzed and plotted using Flowing software (v2.5.1, Cell Imaging and Cytometry Core, Turku Bioscience Centre, Turku, Finland, with the support of Biocenter Finland). Further evaluations were carried out based on the determined geometric mean of the fluorescence intensity.

Statistics

The statistical significance between means was evaluated using Two Sample t-test or one-way ANOVA with subsequent Bonferroni corrected post hoc paired t-test using R software. The data was tested for variance homogeneity using Levene test, before.

Software

The software used for the different experiments is listed below.

TopSpin 4.1.4 software (Bruker Corporation/Billerica/MA/USA, URL: <https://www.bruker.com/en/products-and-solutions/mr/nmr-software/topspin.html>) was used for NMR processing and analysis.

Origin 2022b software (OriginLab Corporation/Northampton/Ma/ USA. URL: <https://www.originlab.com/2022b>) was used for data plotting and analyzing.

Flowing software (v2.5.1, Cell Imaging and Cytometry Core, Turku Bioscience Centre, Turku, Finland, with the support of Biocenter Finland. URL <https://flowingsoftware.com/download/>) was used for FACS data analyses and plotting.

R software (R Core Team (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>) was used for data plotting and statistical analysis.

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Chapter 6

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Supplementary Information

Block Copolymer Synthesis and Characterization

COOH-PEG_{5k}-PLA_{10k} block copolymer was synthesized via ring-opening polymerization of the cyclic lactone 3,6-dimethyl-1,4-dioxane-2,5-dione with the organocatalyst 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) at RT. COOH-PEG_{5k}-OH (Jenkem Technology, Plano, USA, M_n 5000 $\pm$ 500 g mol⁻¹, as stated on certificate of analysis) was used as macroinitiator for the reaction.^[1,2] In brief, the polyethylene glycol (PEG) macroinitiator (1 equiv.) was reacted with 3,6-dimethyl-1,4-dioxane-2,5-dione (70 equiv.) and DBU (3.5 equiv.) in dichloromethane (DCM) as solvent at RT. After 1 h the reaction was quenched with benzoic acid in excess (17.5 equiv.). The resulting block copolymer was precipitated in ice-cold diethyl ether, filtered, and dried under the nitrogen flow. Identity and purity of the block copolymer was verified by a ¹H-NMR spectrum recorded on a Bruker Avance III HD 400 operating at 400 MHz; the block copolymer was dissolved in deuterated chloroform (CDCl₃).^[3] Furthermore, the molecular weight of the synthesized polymer was determined from the ¹H-NMR spectra as previously described by our group.^[4] Topspin 4.1.4 software was used to evaluate the NMR spectra (Chapter 5, Figure S5).

Fluorescence Labeling of PLGA with CY-5

Poly(D,L-lactide-co-glycolide) (PLGA) (Resomer RG 502, carboxylic acid-terminated, M_w 7000-17000, Sigma-Aldrich, St. Louis, USA) was fluorescently labeled by covalent linkage with cyanine-5-amine (Lumiprobe GmbH (Europe), Hannover, Germany). In brief, carboxylic acid-terminated PLGA (1 equiv.) was dissolved together with cyanine-5-amine (CY-5) (0.1 equiv.) in dry *N,N*-dimethylformamide (DMF). The carboxylic acid group was activated by the addition of (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (2 equiv.) and DIPEA (4 equiv.). The reaction mixture was stirred overnight at RT under light protection. To remove the excess of free dye, the polymer was precipitated several times in ice-cold diethyl ether until the supernatant was no longer blue. The product was dried under the nitrogen flow and characterized by FTIR. FTIR spectra were recorded on an Agilent Technologies Cary 630 FTIR (Agilent/ Santa Clara/ USA) with 32 scans, 8 cm⁻¹ resolution (Chapter 5, Figure S4).

MTT Assay

THP-1 monocytes were seeded into 96-well plates at a density of 60,000 cells/well. The monocytes were differentiated into macrophages by addition of 100 nM PMA to the cell culture medium and incubated for 72 h (37 °C, 5 % CO₂) before proceeding the MTT assay.

The cells were incubated under the specified experimental conditions (A-C):

	Treatment	Concentrations	Incubation regime
A	Unmodified, unstained LNCs	3 nM, 1 nM, 0.3 nM	24 h, 1 h, 0.5 h and dual LNC application: 24 h + 2 h
B	ENPs	1 nM, 0.1 nM	24 h, 1 h, 0.5 h
C	Chloroquine diphosphate (aqueous solution in RPMI medium) + ENPs	Chloroquine diphosphate: 200 μ M, 100 μ M ENPs: 0.1 nM	0.5 h preincubation with chloroquine diphosphate and subsequent addition of ENPs for further 4 h 0.5 h preincubation with chloroquine diphosphate and subsequent addition of ENPs for further 2 h

After the incubation period, the samples were discarded and 160 μ L MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent was added and incubated for 3.5 - 5 h. Afterwards, the MTT reagent was aspired and 50 μ L of 10 % sodium dodecyl sulfate (in PBS) was added. The wells were incubated overnight at RT. The results were measured the next day after gentle shaking of the well plates. The absorbance was measured at wavelengths of λ 570 nm and λ 690 nm on a FLUOstar Omega plate-reader (BMG Labtech, Ortenberg, Germany). The viability was assessed calculating the differences in absorbance at both wavelength to the control. Results represented as mean $\pm$ SD (n = 3 for samples and n = 6 for controls).

Calculation of the Required Amount of Chloroquine for in vivo Dosing

To transiently suppress the mononuclear phagocyte system (MPS) of healthy immunocompetent female (C57BL/6) mice, Mills et al.^[5] injected 5 mg Kg⁻¹ chloroquine diphosphate (dissolved in 100 μ L sterile PBS) intravenously via the lateral tail vein 12 h prior to the intravenous administration of hyperbranched polymers (employed as a model nanoparticulate system). To estimate the chloroquine dosing for MPS suppression in immunocompetent healthy male Wistar rats, a dose conversion between species was performed according to equation 1.^[6,7] The K_m factor, which is calculated according to equation 2, was taken from the literature.^[6]

$$\text{Animal A} \left[\frac{\text{mg}}{\text{kg}} \right] = \text{Animal B} \left[\frac{\text{mg}}{\text{kg}} \right] \cdot \frac{K_m \text{ coefficient of Animal B}}{K_m \text{ coefficient of Animal A}}$$

eq. 1

$$K_m = \frac{\text{average body weight [kg]}}{\text{body surface area [m}^2\text{]}}$$

eq. 2

From this, a dose of 2.5 mg Kg⁻¹ was calculated for the treatment of rats. Assuming a maximum weight of 600 g per rat (350-560 g),^[8] this corresponds to a maximum dose of 1.5 mg chloroquine per rat. In the study by Mills et al.,^[5] chloroquine diphosphate was used, whereas in the present study the free chloroquine base was employed for the encapsulation into LNCs. For the dose conversion between the species, the salt was equated with the free base. However, dose optimization, potentially through adjustment of the administration regime (dosing frequency), must be conducted in vivo. It is recommended to administer rats subcutaneously at a maximum dose of 10 mL per kg body weight.^[9] This corresponds to an administrable volume of up to 6 mL per rat (600 g body weight). At a planned application volume of 250 µL, the administered batch must contain a chloroquine concentration of 6 mg mL⁻¹.

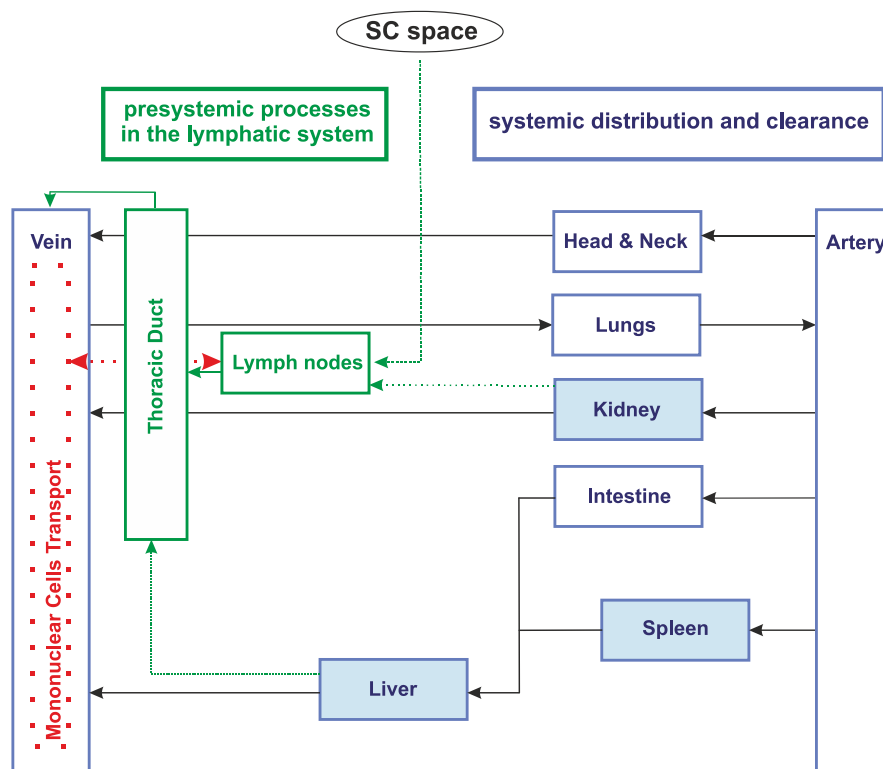


Figure S 1 Schematic illustration of distribution compartments for nanoparticles after subcutaneous administration based on a physiologically based pharmacokinetic (PKPD) model published by Perazzolo et al.^[10] The lymphatic network is depicted in green, while organs and tissues involved in the systemic circulation are represented in blue. Light blue shading denotes organs belonging to the reticuloendothelial system (RES). Arrows represent blood and lymph supplies. The red dotted line and arrows represent the mononuclear cells transport.

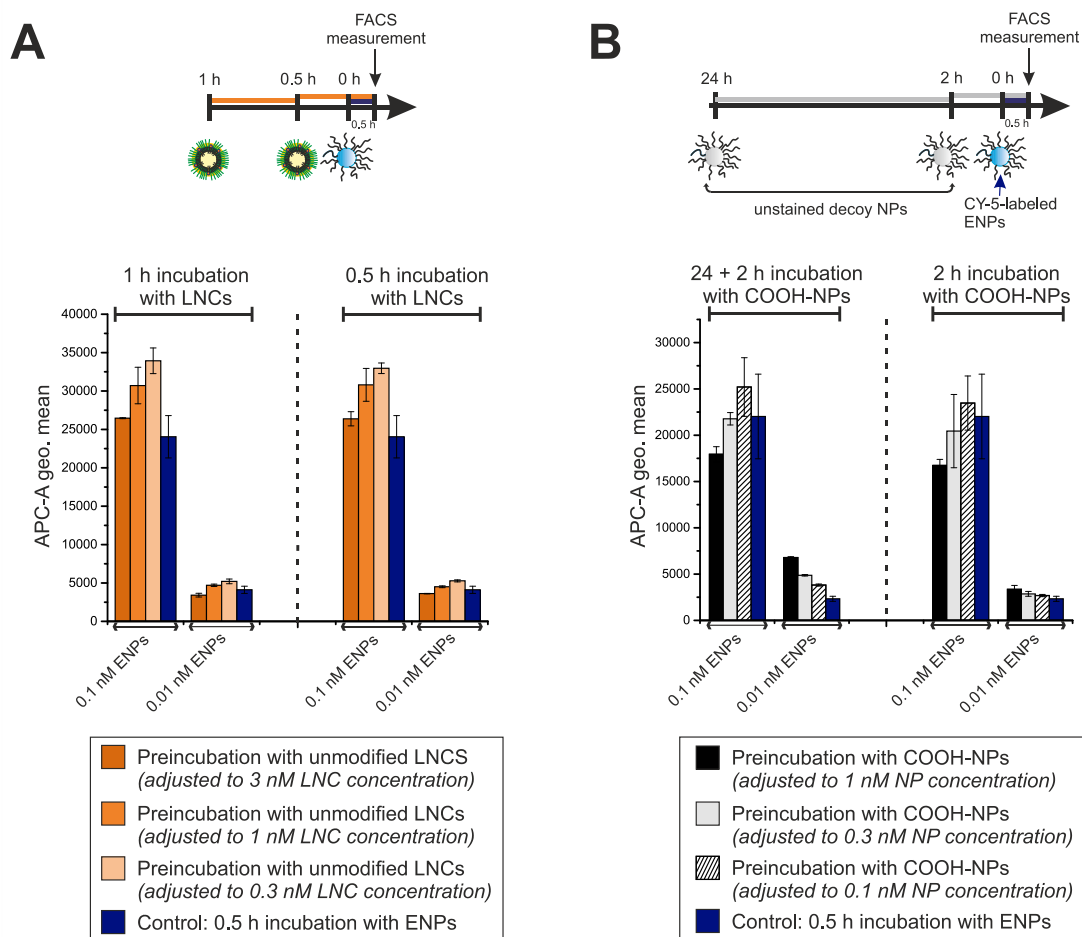


Figure S 2 Evaluation of the unmodified LNCs and unstained polymer nanoparticles (COOH-NPs) as decoy agents. (A) Visualization of the administration regime: The macrophages were preincubated for 1 h or 0.5 h with unmodified LNCs before the CY-5-labeled ENPs were added for a further 0.5 h. Unmodified LNCs were applied at concentrations of 3 nM, 1 nM and 0.3 nM and the ENPs at concentrations of 0.1 nM and 0.01 nM. The mean fluorescence intensity (APC-A geo. mean) was assessed as an indicator for the uptake of ENPs into macrophages after preincubation with unmodified LNCs as decoy agents and quantified using flow cytometry analysis. Results represented as mean $\pm$ SD ($n = 3$). (B) Visualization of the administration regime: The macrophages were pretreated with either a dual application of COOH-NPs, which were applied for 24 h and 2 h, or with a single application of COOH-NPs, which were applied for 2 h, before the ENPs were added for a further 0.5 h. COOH-NPs were applied at concentrations of 1 nM, 0.3 nM and 0.1 nM and the ENPs at concentrations of 0.1 nM and 0.01 nM. Results represented as mean $\pm$ SD ($n = 3$).

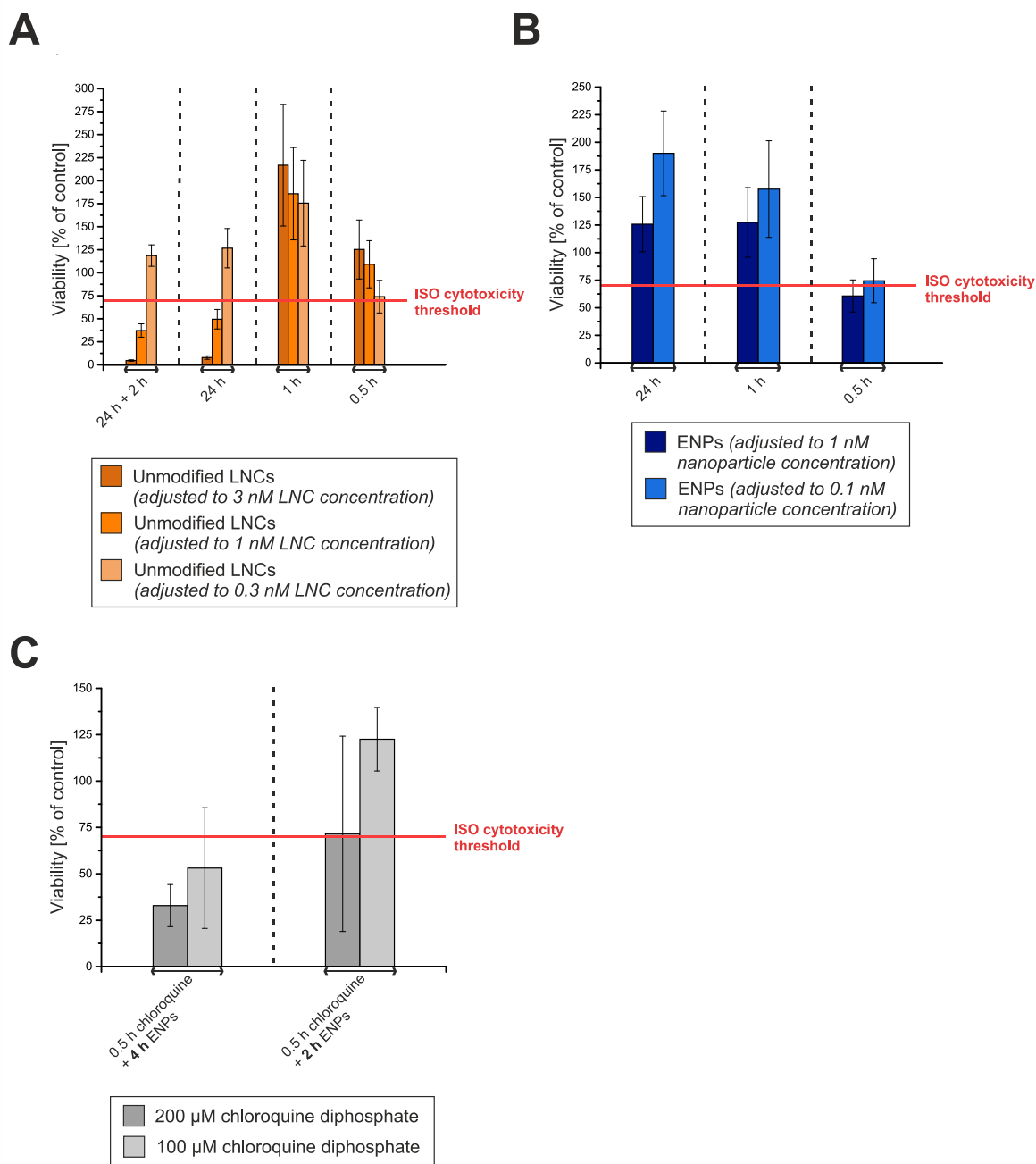


Figure S 3 Assessment of the cytotoxicity under conditions relevant for the FACS experiments. (A) Results of the MTT-assay after incubation of macrophages with unmodified LNCs applied at concentrations of 3 nM, 1 nM and 0.3 nM in a dual application regime at 24 h and 2 h, or for 24 h, 1 h or 0.5 h. Results represented as mean $\pm$ SD ($n = 3$ for samples and $n = 6$ for controls). (B) Results of the MTT-assay after incubation of macrophages with ENPs applied at concentrations of 1 nM and 0.1 nM for 24 h, 1 h or 0.5 h. Results represented as mean $\pm$ SD ($n = 3$ for samples and $n = 6$ for controls). (C) Results of the MTT-assay after preincubation of macrophages with chloroquine diphosphate applied at concentrations of 200 μ M or 100 μ M for 0.5 h and subsequent incubation with ENPs for 4 h or 2 h. Results represented as mean $\pm$ SD ($n = 3$ for samples and $n = 6$ for controls).

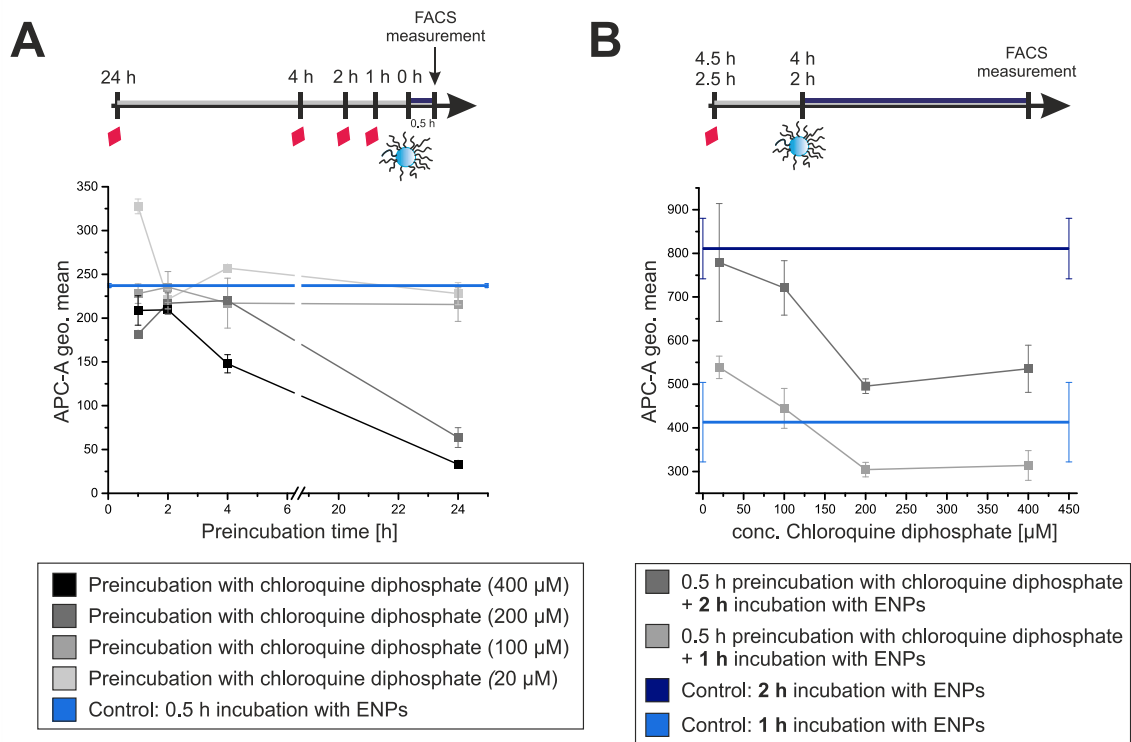


Figure S 4 Evaluation of chloroquine diphosphate as an endocytosis inhibitor. The mean fluorescence intensity (APC-A geo. mean) was assessed as an indicator for the uptake of CY-5-labeled ENPs into macrophages. Results represented as mean $\pm$ SD ($n = 3$). Macrophages, which were treated exclusively with ENPs, without prior incubation with chloroquine diphosphate, served as control (indicated in blue). (A) Visualization of the administration regime: Macrophages were preincubated with chloroquine diphosphate for 24 h to 1 h before the ENPs were added for further 0.5 h. (B) Visualization of the administration regime: Macrophages were preincubated with chloroquine diphosphate for 0.5 h before the ENPs were added for further 4 h or 2 h.

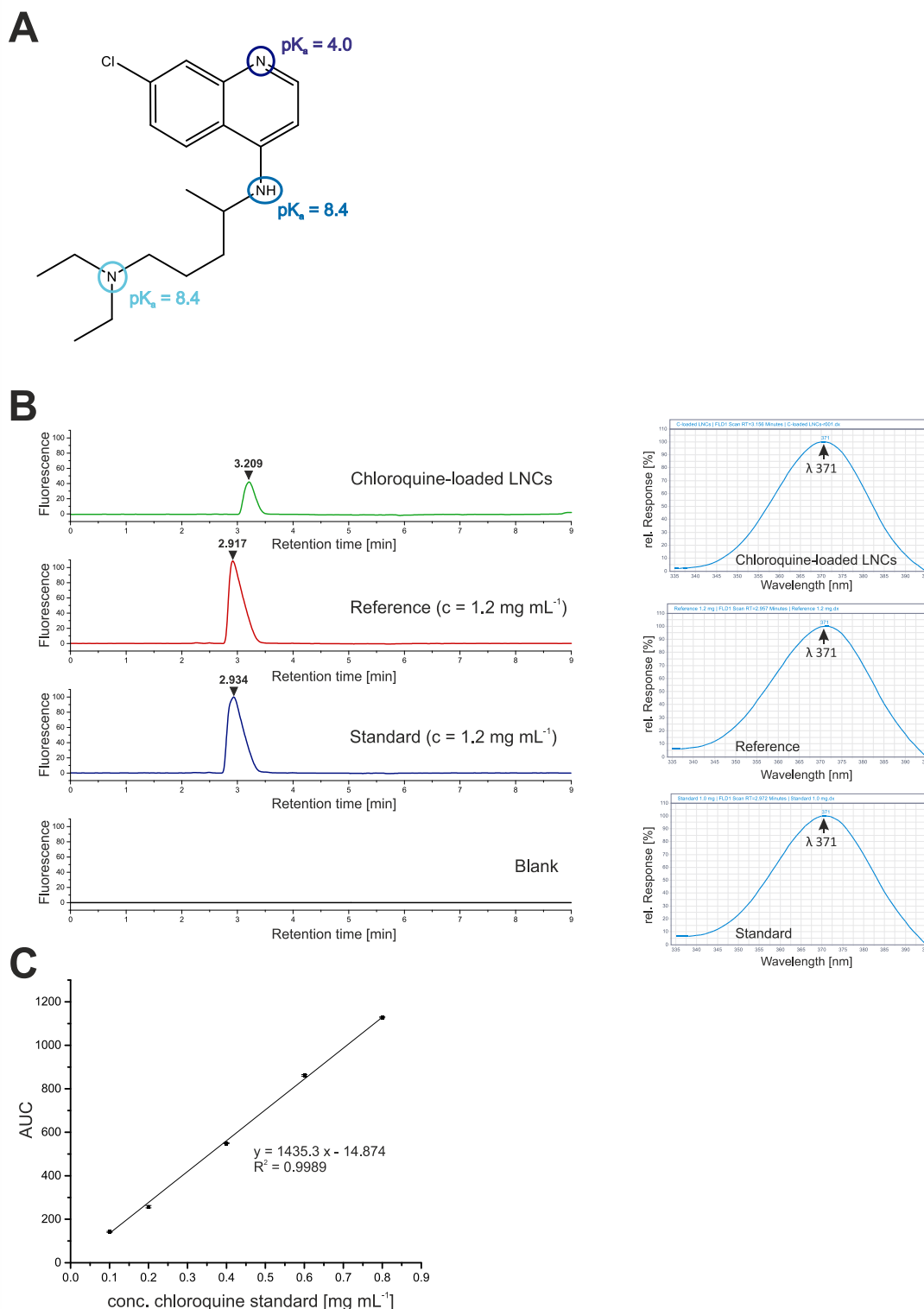


Figure S 5 Chloroquine structure and HPLC analysis results of chloroquine standards, reference and chloroquine-loaded LNCs. (A) Chemical structure of the chloroquine base. (B) HPLC chromatograms of the solvent blank, chloroquine base synthesized from chloroquine diphosphate (standard, $c = 1.2 \text{ mg mL}^{-1}$), chloroquine base purchased from MedChemExpress (Hözel Diagnostika Handels GmbH, Köln, Germany) (reference, $c = 1.2 \text{ mg mL}^{-1}$) and chloroquine-loaded unmodified LNCs. Emission spectra of the peaks. (C) Calibration curve of the area under the curve (AUC), obtained from HPLC analyses, plotted as a function of the chloroquine standard concentration ($R^2 = 0.9989$). Results represented as mean $\pm$ SD ($n = 3$).

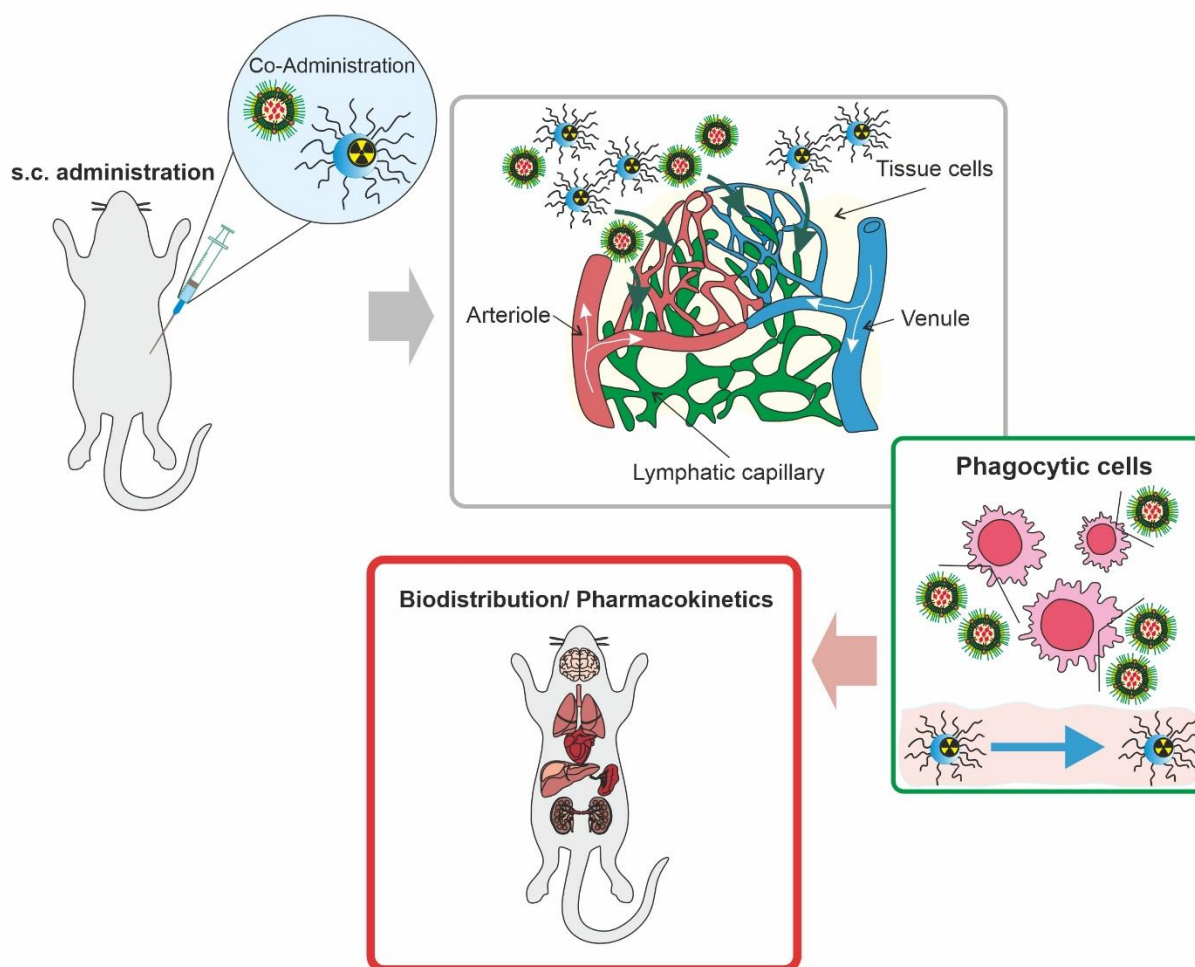
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Outlook

Chapter 6

Chapter 6 outlines the *in vitro* results of the coadministration regime developed to achieve systemically accessible concentrations of ENPs after subcutaneous administration. The *in vitro* study successfully demonstrated that the coadministration of the ENPs with chloroquine-loaded decoy LNCs, resulted in the statistically significant reduced endocytosis of ENPs by macrophages. The obtained results are highly promising for the progression of the study, allowing the identification of three prospective research goals, which are presented and discussed below in their experimentally logical sequence.



Graphical depiction of the outlook.

Goal 1: Evaluating the coadministration regime for the subcutaneous application of ENPs *in vivo*.

Building on the promising results of the *in vitro* study, the next step involves the evaluation of the coadministration regime *in vivo*. Therefore, studies on healthy male Wistar rats are planned in close collaboration with our research partners. The primary outcome to be assessed in these first *in vivo* studies is, whether the ENPs can be detected in measurable concentrations in the systemic circulation following subcutaneous coadministration with chloroquine-loaded decoy LNCs. The

required dose of chloroquine for immunomodulation in rats was calculated based on literature data^[1] using inter-species dose conversion,^[2,3] but has to be validated in vivo. The same holds for the dosing schedule for decoy LNCs and ENPs. If required, adjustments of the overall chloroquine dose or the dosing schedule should be considered. It must be carefully evaluated whether the finally administered dose of chloroquine-loaded LNCs induces toxicity, either through particle-induced effects or the released chloroquine. Chloroquine exhibits a large volume of distribution attributed to its lysosomal trapping.^[4,5] Consequently, the potential accumulation of chloroquine following repeated dosing must be monitored for its effects. Mills et al.^[6] critically assessed that most administration regimes for nanoparticles are solely monitored within very short time frames. Thus, only limited data on repeated dosing schedules or the long-term effects of administration regimes, e.g. adaptation effects of the immune system, are available.^[6] Closing this knowledge gap by evaluating the efficiency of the developed coadministration regime in repeated dosing schedules in vivo offers a promising avenue for further investigations.

To make the ENPs accessible for real-time tracking in vivo via SPECT/CT, they can be labeled with the radionuclide technetium-99m (^{99m}Tc) in addition to their CY-5-tagged core. In a previous study (Chapter 5), we have successfully demonstrated that ^{99m}Tc can be directly incorporated into the core/shell structure of the nanoparticles without introducing additional chelating groups into the structure. However, due to the short half-life of ^{99m}Tc of 6.02 hours,^[7] the observation period is limited to 24 hours. Replacing ^{99m}Tc with indium-111 (¹¹¹In), which has a half-life of 2.83 days,^[8] is theoretically feasible, but not practicable due to the limited availability of ¹¹¹In. Although the established ENPs ($dh < 100$ nm) can be absorbed from the subcutaneous tissue via the lymph vessels, initial in vivo results revealed that the kinetics of this process extend beyond the feasible observation period of 24 hours. To accelerate the uptake of the ENPs, their hydrodynamic diameter must be reduced to a size range of 20 nm to 50 nm.^[9] In a previously published study by our group, it has been successfully demonstrated that polymer nanoparticles can be produced within a size range of 30 nm to 40 nm with a narrow PDI using microfluidics.^[10] Recent experiments using the Nanoassembler Ignite® (Precision NanoSystems, Vancouver, Canada) for ENP production showed that these values are also reproducible for CY-5-labeled core-shell nanoparticles. Further in vivo experiments are therefore planned with ENPs produced using microfluidics.

One question that has not been considered in previously published studies on radiolabeled nanoparticles is, whether the radiolabel remains associated with the nanoparticles after in vivo administration. This question becomes particularly relevant when the radiolabel is merely integrated into the structure of the nanoparticles. So far, this aspect has only been assessed in vitro using stability and challenge tests with chelating agents, e.g. DTPA or cysteine.^[11–13] In close collaboration with our partners, it is therefore planned to establish methods to evaluate the integrity of the unit of radiolabel and nanoparticles following in vivo administration over relevant measurement periods. A particular challenge in this process is the extraction of the polymer core-shell nanoparticles from the tissue and their separation from the protein corona. The nanoparticles are acid-labile^[14,15] and lose their integrity in organic solvents.^[13] Therefore, only enzymatic tissue digestion^[16] is considered

practicable for their extraction. It is planned to establish a protocol, in which enzymatically digested liver homogenate, obtained from rats previously treated with ^{99m}Tc -labeled nanoparticles, is analyzed using SDS-PAGE. Due to the selected mesh size of the polyacrylamide gel, the ^{99m}Tc -labeled nanoparticles are expected to remain within the pockets of the gel, while the proteins are separated. Furthermore, as an orthogonal approach, co-localization studies between the radiolabel and the fluorescence label are intended to provide insights into the integrity of the unit of radiolabel and nanoparticles. For this purpose, methods must be transferred and established between the laboratories to preserve the fluorescence signal in the tissue beyond the decay period of the radiolabel and to prepare tissue sections.

Goal 2: Analyzing the biodistribution and pharmacokinetics of the ENPs in vivo.

A further research objective could be the systematic investigation of the biodistribution and pharmacokinetic (PK) profiles of the ENPs following subcutaneous administration in comparison to an intravenous administration regime. Previous studies on the biodistribution and PK profiles of nanoparticles concluded that a fundamental transferability of the results between different nanoparticle types is not feasible.^[17,18] The obtained results were dependent on the nanoparticle size, their surface decoration, their zeta potential, as well as methodological factors such as the detection methods, administration routes and the selected animal model.^[17,18] Upon reaching the systemic circulation, the transport of nanoparticles into tissues is typically concentration dependent and exhibits saturability.^[17,18] Nanoparticle aggregation and accumulation in vivo, along with their opsonization and the formation of a protein corona can influence their PK and biodistribution profiles.^[17–20] Currently, insufficient data is available to accurately estimate the effect resulting from the interplay of these individual factors. Consequently, physiologically based pharmacokinetic (PBPK) modelling for nanoparticles is challenging. Their in vivo transport mechanisms are more complex than those of small molecules and furthermore influenced by various factors, such as their uptake by the MPS, their enzymatic degradation and the change of their physical properties.^[18,21] Additionally, ligand-functionalized nanoparticles can undergo target-mediated deposition affecting their PK and biodistribution profiles.^[18] Li et al.^[21] conducted a PBPK study on PLGA nanoparticles following intravenous administration. They examined the effect on varying PEG contents in the nanoparticle structure. Due to the similar structure of the analyzed nanoparticle, this study could serve as reference for the core-shell ENPs we established, at least for intravenous administration regimes. However, due to the limited transferability, conducting a dedicated biodistribution and PK study would be of considerable interest. This could be linked to investigations of whether the expected retardation following the subcutaneous administration of the nanoparticles contributes to an altered biodistribution. It would be expected that their sustained release leads to a reduced nanoparticle-induced toxicity in the off-target organs, e.g. liver and spleen. Furthermore, it would be of interest whether the nanoparticle release from the subcutaneous tissue and their plasma concentration could be regulated by adjusting the size of the ENPs relative to the size of the co-applied decoy LNCs.

Goal 3: Incorporating targeting-sequences into the structure of the ENPs.

The therapeutic efficacy of the ENPs as drug delivery systems is critically dependent on their ability to reach their targets in therapeutic relevant concentrations. To characterize the therapeutic efficiency of the ENPs following the described subcutaneous coadministration regime, it will be necessary to implement a targeting sequence into the structure of the ENPs to induce their tropism toward target cells or tissues. In a previously published study by our group, structurally equivalent core-shell nanoparticles were functionalized with two ligands in an adenovirus-mimetic design to target kidney mesangial cells.^[22] The nanoparticles were loaded with erastin, a ferroptosis inducer, and administered intravenously to mice. Based on the observed ferroptosis-dependent cellular damage in the kidney mesangium after 10 days, it was successfully demonstrated that the errastin-loaded nanoparticles reached the targeted tissue in concentrations relevant to the intended biological effect. A similar experimental setup could also be employed to assess whether nanoparticles, following subcutaneous administration, reach their targets in therapeutically relevant concentrations. However, to comprehensively characterize their targeting efficiency, it will be crucial to further evaluate their biodistribution and the potential nanoparticle-induced damage occurring in liver and spleen,^[23,24] which represent the main organs involved in the metabolism of nanoparticles.^[1,25,26]

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Chapter 7

Summary

Although nanoparticles have been intensively studied as promising drug delivery systems for decades, their transfer from bench to bedside is rarely achieved.^[1,2] One key reason for this is the insufficient bioavailability and, consequently, poor targeting efficiency of nanoparticles due to their rapid recognition and clearance by the immune system following *in vivo* administration.^[3–5] To address this challenge and to enhance their targeting efficiency, strong emphasis has been placed on optimizing the physicochemical properties of nanoparticles.^[6,7] A major focus was on the development of increasingly complex targeting strategies by functionalizing their surfaces with ligands as targeting moieties.^[8] However, current knowledge gaps in the understanding of the interaction between engineered nanoparticles and their target cells limit the effective translation into a rational nanoparticle design. The lock-and-key model, originally introduced by Emil Fischer,^[9] is frequently employed to describe these interactions. Thereby, the “lock” refers to the target cell receptors and the “key” to the targeting moieties, respective ligands, on the nanoparticles’ surface. This, however, represents a simplification that does not fully capture the complexity of the nanoparticle-cell interactions (**Chapter 1**). The binding of engineered nanoparticles toward the cell surface is composed of specific ligand-mediated binding events and is furthermore non-specifically driven by their high surface energy. Moreover, the multivalent nature of binding leads to superordinate binding motives on both the structuring of the nanoparticles, and, responsively, on the cell surface. To optimize ligand-functionalized nanoparticles in terms of the selectivity and specificity of their binding, consequently, a more comprehensive understanding of their interactions with cells must be developed.

With the aim of contributing to a systematic understanding of the interaction between engineered nanoparticles and their target cells, the quantity of specific ligand-receptor interactions per nanoparticle was examined as a decisive factor for selectivity (**Chapter 3**). Compared to viruses, which are adorned with an evolutionarily optimized number of binding-domain carrying spikes on their surface, nanoparticles are, on average, equipped with significantly more ligands.^[10] To determine the fraction of specifically binding ligands in the densely functionalized nanoparticle corona, an experimental approach was presented. EXP3174-functionalized core-shell nanoparticles were employed as a model system. Among a total ligand valence of 29 ligands/100 nm² on their surface, only a minor fraction of 5.3 ligands/100 nm² was demonstrated to bind specifically to target cell receptors. By this, it was successfully proven that the number of specifically binding ligands on the nanoparticles’ surface is of the same order of magnitude as the spike density of viruses. This strongly underlines the fact, that the selectivity of the nanoparticles is weakened by their high ligand valence. By considering the receptor structure and surface morphology of the employed target cell line, the obtained result was furthermore validated for plausibility in the biological context. The targeted angiotensin-II type 1 receptor is frequently organized into microdomains as clusters. This makes it plausible that each nanoparticle can bind on average a maximum of 251 receptors on the target cell surface. These findings underscore the need to design the surface-functionalization of nanoparticles based on a more thorough understanding of the structuring of their target cells to achieve binding selectivity. In contrast, simply reducing the ligand valence to the number of

specifically binding ligands does not seem to be promising, as the ligand-bearing polymer chains, unlike viral spikes, are not spatial mobile and therefore cannot locally condense.

Furthermore, the potential of the engineered model nanoparticles to interact non-specifically with their target cells was investigated (**Chapter 4**). The charge of the spacer chain head groups was examined as a potential influencing factor for non-specific adhesion to biomembranes. Therefore, binding studies with differently charged nanoparticles were conducted on target cells. However, the obtained results remained inconclusive. Despite statistically significant different binding behavior as a function of the nanoparticles' surface charge, the determined effect size called the biological relevance of the obtained results into question. Additionally, the ligand EXP3174 was analyzed to promote non-specific interaction with the cell membrane due to its high hydrophobicity. It is known that at least one binding mechanism of the ligand with the receptor is based on 2D diffusion in the cell membrane. The hypothesis was examined whether the functionalization of hydrophilic PEG chains with the hydrophobic ligand could lead to the formation to a reverse surfactant-like structure, well suited to intercalate into biomembranes. Therefore, binding studies on both, target cells and supported lipid bilayers, were conducted, which, however, failed to confirm the hypothesis. The results, consequently, emphasize that that each nanoparticle system must be assessed individually for its potential to engage in non-specific interactions with cells.

To record the biodistribution of the polymer model nanoparticles, they were made accessible for real-time detection in vivo by embedding technetium-99m into their structure (**Chapter 5**). Unfunctionalized nanoparticles were employed to record a blank for the distribution of the entire core-shell nanoparticle platform, ensuring that it remains unaffected by effects from the ligand. During the examination of the radiochemical purity of the radiolabeled nanoparticles, it was observed that previously published purity assessments using thin-layer chromatography did not accurately reflect the composition of the batches. Radioactive agglomerates could not be clearly distinguished from radiolabeled nanoparticles in the analysis. The application of contaminated batches, however, can have severe consequences for the interpretation of the obtained in vivo results. Therefore, systematic investigations on the universal applicability of conventional thin-layer chromatography for comprehensively assessing the radiochemical purity of polymer nanoparticles were conducted. The findings indicate that the complex and heterogeneous composition of polymer nanoparticles hinders the development of universally applicable methods and the transferability of thin-layer chromatography as a standalone quality control tool between different nanoparticle types.

Following in vivo administration, nanoparticles are recognized and sequestered by cells of the immune system, before they can bind with their engineered surfaces to target cells. Thus, relying exclusively on a material science-based approach – by optimizing their surface design – does not adequately address the challenge of the nanoparticles' poor targeting efficiency. A complementary strategy is to influence the distribution of the nanoparticles between target and off-target cells and, consequently, their targeting efficiency through a temporary immunomodulation of the organism. To accomplish this, a coadministration regime with decoy particles was developed to inhibit the uptake of the effector nanoparticles into off-target immune cells (**Chapter 6**). Both, polymer and lipid

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particles were tested for their ability to saturate the uptake capacity of macrophages, which were employed as cell model for immune cells. Furthermore, chloroquine, an endocytosis inhibitor,^[11] was encapsulated into lipid nanocapsules to achieve uptake inhibition for the effector nanoparticles. This coadministration regime was developed to achieve systemic availability of the nanoparticles after subcutaneous administration, thereby opening a new application route for nanoparticles. It was successfully demonstrated in vitro that preincubation of immune cells with chloroquine-loaded decoy lipid nanocapsules led to a statistically significant reduction of the endocytosis of the subsequently applied effector nanoparticles. These results should be validated in future in vivo studies.

Conclusion

In the present work, the binding of ligand-functionalized polymer nanoparticles toward their target cells was successfully characterized in terms of specific and non-specific interactions. The number of ligand-receptor interactions was determined to an average maximum of 251 binding ligands per nanoparticle. With this, it was clearly demonstrated that only a minor fraction of the ligands on the nanoparticle surface were involved in specific interactions with target receptors. The result was discussed as a decisive factor for binding selectivity in comparison to the evolutionary optimized spike density of viruses. Together with studies on influencing factors on the non-specific binding of the nanoparticles, these investigations aimed at contributing to a better understanding of the nanoparticle-cell binding. They consequently provide the basis for rational design strategies for nanoparticles. However, to increase the targeting efficiency of nanoparticles *in vivo*, comprehensive approaches to the engineering-based strategies must be developed. The nanoparticles must be shielded from immune system recognition to facilitate their specific interaction with target cells. To accomplish this, a coadministration regime was developed to saturate the uptake capacity of immune cells. The aim was to protect the nanoparticles from clearance, while making them accessible for non-systemic administration routes. *In vitro*, it was successfully demonstrated that preincubation of immune cells with chloroquine-loaded decoy particles led to a significant reduction of the endocytosis of subsequently applied nanoparticles. In future studies, the efficacy of the coadministration regime is to be validated.

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Appendix

Abbreviations

<i>A1</i>	Baseline in the dose-response model
<i>A2</i>	Maximum response asymptote in the dose-response model
ACD solution	Solution of 0.068 M citrate, 0.074 M dextrose, adjusted to pH 5
ACN	Acetonitrile
AT1R	Angiotensin-II type 1 receptor
BNP	Bystander nanoparticles
<i>B_{max}</i>	Maximal binding capacity
BSA	Bovine serum albumin
CCP	Clathrin coated pit
CDCl ₃	Deuterated Chloroform
CLSM	Confocal laser scanning microscopy
COOH-NPs	Unfunctionalized polymer core-shell nanoparticles carrying carboxy-terminated PEG-chains in their shell
CY-5	Cyanine-5-amine
DAPI	4',6-Diamidino-2-phenylindole
DBCO	Dibenzocyclooctyne
DBU	1,8-Diazabicyclo(5.4.0)undec-7-ene
DCM	Dichloromethane

DIN EN ISO	Recognized standard in Germany, Europe and internationally
DiO	3,3-Dioctadecyloxacarbocyanine
DIPEA	<i>N,N</i> -Diisopropylethylamine
DLS	Dynamic light scattering
DMF	<i>N,N</i> -Dimethylformamide
DMSO	Dimethyl sulfoxide
DMSO-d ₆	Deuterated dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
DOPC	1,2-Dioleoyl- <i>sn</i> -glycero-3-phosphocholine
DOPS	1,2-Dioleoyl- <i>sn</i> -glycero-3-phosphoserine
100 %-DOPS-LNCs	LNCs, in which the DOPC is quantitatively substituted with DOPS
<i>E</i>	Effect at the receptor
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
<i>EE</i>	Encapsulation efficiency
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol-bis(β-aminoethyl ether)- <i>N,N,N',N'</i> -tetraacetic acid
ENP	Effector nanoparticle
<i>E_{max}</i>	Maximal effect at the receptor

Appendix

Equiv.	Equivalent
ESI	Electrospray ionization source
EXP3174	Losartan carboxylic acid
25 %-EXP-NPs	Nanoparticles carrying 25 % EXP3174-functionalized chains in their shell and carboxy-terminated spacer chains
25 %-EXP-NPs_selenium-containing	Selenium-tagged nanoparticles carrying 25 % EXP3174-functionalized chains in their shell and carboxy-terminated spacer chains
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FDA	Food and Drug Administration
Fluram	Fluorescamine, 4'-Phenylspiro[2-benzofuran-3,2'-furan]-1,3'-dione
FNP	Functionalized nanoparticles
FTIR	Fourier-transformed infrared spectroscopy
G	Unit for relative centrifugal force
GFP	Green fluorescent protein
Gp	Glycoprotein
h	Hour
HBTU	(2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
He-Ne laser	Helium-neon laser

^1H -NMR	Hydrogen-1 nuclear magnetic resonance spectroscopy
HPLC	High-performance liquid chromatography
HR- $^{99\text{m}}\text{Tc}$	Hydrolyzed-reduced technetium-99m
[^3H]UR-MK292	Tritium labeled angiotensin-II derivative
%ID/g	Percentage of injected dose per gram of tissue
IC_{50}	Half maximal inhibitory concentration
ICP-OES	Inductively coupled plasma optical spectrometry
ITS	Insulin-transferrin-selenium
i.v.	Intravenous
K_D	Equilibrium dissociation constant
kDa	Kilodalton
K_i	Equilibrium inhibition constant
LNCs	Lipid nanocapsules
LoQ	Limit of quantification
Lys-Ang-II	Lys-angiotensin-II (Sequence: $\text{NH}_2\text{-KDRVYIHPF-COOH}$)
Milli-Q water	Ultrapure water
min	Minutes
$^{99}\text{Mo}/^{99\text{m}}\text{Tc}$ generators	Molybdenum-99/technetium-99m generator
mPEG $_{5K}$	Poly(ethylene glycole) monomethyl ether with an average molecular weight of 5000 g mol^{-1}

Appendix

MPS	Mononuclear phagocyte system
M_w	Molecular weight
MWCO	Molecular weight cut-off
N_{AT1R}	Number of available AT1 receptors
N_{bound}	Number of specifically binding ligands per nanoparticle
NHS	N-Hydroxysuccinimide
N_{NP}	Number of nanoparticles attached to the cell membrane
N_{OC}	Number of occupied receptors
NPs	Nanoparticles
NTA	Single nanoparticle tracking analysis
p	Slope in the dose-response model
PALM	Photoactivation localization microscopy
PDI	Polydispersity index
PEG	Polyethylene glycole
PEG- <i>b</i> -PPS	poly(ethylene glycol)- <i>block</i> -poly(propylene sulfide)
PEI	Polyethylenimine
PFA	Paraformaldehyde
PICALM	Phosphatidylinositol binding clathrin assembly protein
PLA-PEG	Poly(ethylene glycol)- <i>b</i> -poly(D,L-lactide)
PLGA	Poly(D,L-lactide-co-glycolide)

PMA	Phorbol-12-myristate-13-acetate
ppb	Parts per billion
PS	Polystyrene nanoparticles
Py/AA/H ₂ O	Pyridine/acetic acid/water (3:5:1.5, v/v)
Q-Dot	Quantum dot
Q-TOF	Quadrupole time-of-flight
RIfS	Reflectometric interference spectroscopy
rMC	Rat mesangial cells
R _{max}	Maximum ratio in the Grynkiewicz-equation
R _{min}	Minimum ratio in the Grynkiewicz-equation
rpm	Rotations per minute
RT	Room temperature
s.c.	Subcutaneous
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sec	Seconds
SLB	Supported lipid bilayer
SPECT/CT	Single-photon emission computed tomography/ computer tomography
SPECT	Single-photon emission computed tomography
SUV	Small unilamellar vesicles

Appendix

TAT	Transactivator of transcription
TC-treated	Tissue culture treated
TEM	Transmission electron microscopy
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
$^{99m}\text{Tc-NPs}$	Technetium-labeled polymer nanoparticles
$^{99m}\text{TcO}_4^-$	Pertechnetate
UHPLC	Ultra-high performance liquid chromatography
ZW2-	4-(Dimethylamino)butyric amid (as head group synthesized on PEG _{5K} -PLA _{10K} chains)
ZW3-	Zwitterionic structure, sulfobetaine (as head group synthesized on PEG _{5K} -PLA _{10K} chains)
ZW4-	4-(Dimethylamino)butyric amid (as head group synthesized on PEG _{5K} chains)
ZW5-	Zwitterionic structure, sulfobetaine (as head group synthesized on PEG _{5K} chains)
ZW6-	Sulfobetaine

Curriculum Vitae

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Scientific education

09/2021 - 05/2025	PhD Research Fellow Department of Pharmaceutical Technology, University of Regensburg
11/2020 - 05/2021	Research Fellow Division of Pharmaceutical Technology FAU Erlangen-Nürnberg
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05/2020 - 10/2020	Postgraduate education as a pharmacist at a hospital pharmacy Krankenhausapotheke Stiftung Kreuznacher Diakonie, Bad Kreuznach
11/2019 - 04/2020	Postgraduate education as a pharmacist at a community pharmacy Apotheke am Bahnhof, Bad Kreuznach
10/2015 - 10/2019	Study of Pharmaceutical Sciences Julius-Maximilians University of Würzburg

School education

2015	A-Levels/ Abitur
2006 - 2015	Grammar school Gymnasium an der Stadtmauer, Bad Kreuznach
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List of Publications

Peer-reviewed publications

1. K. Schorr, S. Beck, O. Zimmer, F. J. Baumann, M. Keller, R. Witzgall, A. Goepferich: The Quantity of Ligand-Receptor Interactions between Nanoparticles and Target Cells. In: *Nanoscale Horizons* 2025, 10 (4), 803-823 (**Chapter 3**).
2. K. Schorr, X. Chen, T. Sasaki, A.-P. Arias-Loza, J. Lang, T. Higuchi, A. Goepferich: Rethinking Thin-Layer Chromatography for Screening Technetium-99m Radiolabeled Polymer Nanoparticles. In: *ACS pharmacology & translational science* 2024, 7 (9), 2604-2611 (**Chapter 5**).
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Conference abstracts

1. K. Schorr, S. Beck, M. Keller, A. Goepferich: Considerations of the Interaction of Virus-mimetic Nanoparticles with Target Cells from a Quantitative Point of View. CRS Germany Local Chapter Meeting, Bad Dürkheim, Germany, 2024.
2. K. Schorr, S. Beck, M. Keller, A. Goepferich: Experimental Approach for the Quantitative Characterization of Multivalent Ligand-Receptor Interactions of Polymeric Nanoparticles with Target Cells. CLINAM, Basel, Switzerland, 2023.
3. K. Schorr, M. Keller, A. Goepferich: Quantitative Characterization of Multivalent Ligand-Receptor Interactions of Polymeric Nanoparticles with Target Cells. Spring School in Medicinal Chemistry, Regensburg, Germany, 2023.
4. K. Schorr, S. Beck, A. Goepferich: Qualitative and Quantitative Characterization of Multivalent Ligand-Receptor Interactions of Polymeric Nanoparticles with Target Cells. CRS Germany Local Chapter Meeting, Würzburg, Germany, 2023.

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Declaration in Lieu of an Oath

I hereby declare that I have completed the dissertation present without the impermissible help of third parties, without the use of resources other than those indicated, and that any data and concepts stemming directly or indirectly from other sources are indicated with citations to the literature.

No further persons were involved with the creation of the contents of the dissertation presented. In particular, I have not made use of the assistance of a doctoral consultant or other person in return for payment. No-one has received payment in kind either directly or indirectly for work which is associated with the content of the dissertation submitted.

The dissertation has not been submitted in the same or similar form to another examining authority, neither in Germany nor abroad.

8 July 2025, Regensburg

Kathrin Schorr