



Development of a highly sensitive photometric readout platform technology using SPAAC-modified HRP liposomes

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Abstract

Liposome-based bioassays take advantage of liposomes' immense signaling power and easy surface modification. Photometric, electrochemical, and fluorescent detection methods dominate their application in bioassays. Here, it is studied how covalent modification of the liposome surface with proteins affects the functionality of entrapped enzymes. Horseradish peroxidase (HRP) was selected as a model for entrapment while streptavidin served as a model surface ligand. Several coupling reagents negatively affected the functionality of entrapped HRP. In the case of EDC-sNHS chemistry, membrane-permeable reagents inactivated HRP. Similarly, when applying copper-free click chemistry, in particular strain-promoted azide-alkyne cycloaddition (SPAAC), azide-functionalized lipids caused enzyme inactivation probably through coordination with the prosthetic heme iron of HRP. In contrast, the inverse approach employing DBCO-functionalized lipids fully preserved HRP activity. Under optimized conditions with coupling times of only 24 h at room temperature, streptavidin coupling efficiencies reached standard levels. Entrapped HRP was stabilized with 310 mM trehalose and 2 mg/mL HSA enabling storage for up to 6 months after streptavidin modification. HRP-loaded liposomes were evaluated by adapting an assay for the detection of SARS-CoV-2 neutralizing antibodies in serum. Therefore, the liposome surface was modified with the receptor binding domain (RBD) of the SARS-CoV-2 spike protein. The RBD-modified liposomes with simple photometric detection demonstrated a similar sensitivity as fluorescent sulforhodamine B (SRB) liposomes, with a strong correlation of both methods (Pearson's $r = 0.987$). HRP liposomes are hence a reliable platform for visual or photometric bioassays providing similar limits of detection as fluorescent approaches making them ideal for routine lab and point-of-care applications.

Keywords Liposomes · Click chemistry · Photometric · Heterogenous assay · Neutralizing antibodies

Introduction

The amphiphilic character of phospholipids leads to their spontaneous self-association into spherical bilayer vesicles when they are hydrated or exposed to an aqueous phase [1]. These vesicles, so-called liposomes, were originally discovered by Bangham and Horne in 1963 [2]. Due to entropic driving forces, the hydrophobic, nonpolar fatty acyl chains of the phospholipids are directed inwards, whereas the polar head groups point outwards and are in contact with the outer or inner aqueous phase [3]. The lipid composition, including the fatty acyl chain length and the degree of saturation, the

cholesterol content, as well as the charge and functionalization of the lipid head groups, influences the bilayer properties such as membrane fluidity, size, stability, rigidity, phase behavior, and elasticity [4, 5]. The most commonly used lipids for liposomes include cholesterol, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylglycerol (PG) [6, 7]. Liposomes are widely applied in biosensing due to their high surface area, large inner volume, biocompatibility, biodegradability, and the easy conjugation of various recognition elements to the liposomal surface. Furthermore, many different signaling agents can be encapsulated such as fluorescent dyes, electrochemical or chemiluminescent markers, enzymes, enzyme substrates, metals, or radioactive isotopes allowing for different read-out strategies and thus a multitude of applications [7–9]. In addition to their use in bioanalytical chemistry, they are widely applied in biochemistry and biology [4], cosmetics [10], food technology [11], drug delivery [12], or

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clinical applications [13]. One of the most commonly used read-out strategies is fluorescence which is known for its excellent sensitivity and reliability [9, 14]. Despite its high sensitivity, fluorescence detection is associated with several limitations such as the risk of false positives due to increased background signals from endogenous fluorescent molecules within the sample matrix and the requirement of expensive instrumentation and hence limited availability in smaller laboratories or point-of-care settings [15, 16]. In contrast, colorimetric detection is a simpler, rapid, and typically more cost-effective alternative to fluorescence-based methods [17] albeit with lower sensitivity [14]. One strategy to overcome this limitation is the use of liposomes, which can encapsulate several signaling molecules, thereby providing substantial signal amplification [18]. Further amplification can be achieved by encapsulating enzymes, which catalytically convert numerous substrate molecules. By choosing a gentle synthesis route and lipids with low phase transition temperatures, various examples have already been published for liposomes entrapping enzymes including amyloglucosidase [19], horseradish peroxidase (HRP) [20–22] glucose oxidase [23], alkaline phosphatase [24], glucose-6-phosphate dehydrogenase [25, 26], and acetylcholinesterase [27, 28].

For the use of liposomes in bioanalysis, their surface must be modified with ligands, biorecognition elements or small molecules. Design considerations typically include the coupling efficiency, conditions maintaining liposome integrity and stability, and effects on the functionality and stability of the surface ligands. In the case of liposomes entrapping enzyme molecules, their stability and functionality must also be taken into account since coupling reagents may penetrate the lipid bilayer and adversely affect enzyme activity. Hence, for the here studied liposomes entrapping HRP, mild bioconjugation strategies need to be chosen to avoid denaturation of the encapsulated enzyme while also ensuring biofunctionality of the coupled protein on the liposomal surface. In general, several coupling strategies for the post-functionalization of the liposomal surface have been established to date, including EDC-sNHS chemistry, Michael addition reactions, thiol-maleimide conjugation, biotin-streptavidin binding, and click chemistry [4, 29–31]. Among these, click reactions comprise a class of highly selective and efficient reactions that enable the reliable coupling of two molecules under mild reaction conditions. These reactions are characterized by stereospecificity, simplicity, and the formation of stable reaction products, while generating minimal byproducts that can be readily removed [32]. Several reaction classes fall into this definition, including cycloadditions of unsaturated species, nucleophilic substitution reactions, Michael additions, or the copper-catalyzed azide-alkyne cycloaddition (CuAAC) [32, 33]. In particular, CuAAC has been extensively studied, but the required metal catalyst restricts its application in biological systems because of the cytotoxicity

of copper ions [34]. Instead, C. R. Bertozzi developed a method which employs a ring-strained alkyne circumventing the use of a metal catalyst relying on a strain-promoted azide-alkyne cycloaddition (SPAAC) [35]. The ring strain lowers the activation energy allowing a reaction under physiological conditions without the need for copper and hence shows no cellular toxicity [35, 36]. By modifying the ring-strained alkyne with electron-withdrawing groups, the reaction can be accelerated [36]. Prominent examples include dibenzocyclooctyne (DBCO) and difluorinated cyclooctyne (DIFO), which have an approximately 60-fold higher reaction rate [34, 36]. Despite its higher reactivity, DBCO is described to be sufficiently stable in aqueous solutions, making it particularly attractive for bioconjugation reactions [37]. In 2006, the group of F. Schuber was the first to use the Cu(I)-catalyzed azide-alkyne reaction for the surface modification of liposomes [38]. Subsequent studies revealed that the use of azide-functionalized lipids is less efficient than the inverse approach when employing CuAAC [8]. To avoid the known toxicity problem, the SPAAC reaction for lipid bilayers has also been extensively studied [30, 39–42]. Interestingly, while most reactions are slower on the liposomal surface than in solution, the SPAAC reaction shows the inverse trend for ligands with low solubility due to beneficial interactions with the lipids, highlighting its use for modification of liposomes [8]. Nevertheless, the SPAAC reaction on liposome surfaces usually requires reaction times of several days to achieve coupling yields exceeding 80% [8, 30, 39].

Here, we studied how the covalent modification of the liposome surface with proteins may affect the activity of entrapped enzyme molecules. Therefore, standard EDC-sNHS coupling and the copper-free click chemistry SPAAC were applied and compared (Fig. 1). For EDC-sNHS chemistry, carboxyl-functionalized liposomes were employed for conjugation to proteins via their amine residues. In contrast, two inverse approaches were studied using SPAAC: a cyclic alkyne moiety is included in the liposomal bilayer while the protein was functionalized with an azide moiety or vice versa. For the entrapment into liposomes, HRP was chosen as model enzyme due to its widespread use as a reporter in immunoassays. Streptavidin was selected as a model protein for surface modification because of the frequently applied streptavidin-biotin interaction. The encapsulated enzyme HRP enables the use of a simple photometric detection with excellent sensitivity. Using enzymes in liposomes exploits a double signal amplification, as the enzymes catalyze the conversion of multiple substrate molecules, providing a second signal amplification alongside the initial amplification provided by the liposomes encapsulating several molecules. In addition to bioanalytical assays, a modification technique for liposomes that ensures the correct folding and activity of the encapsulated protein is required for various applications, such as drug delivery. In this case, liposomes

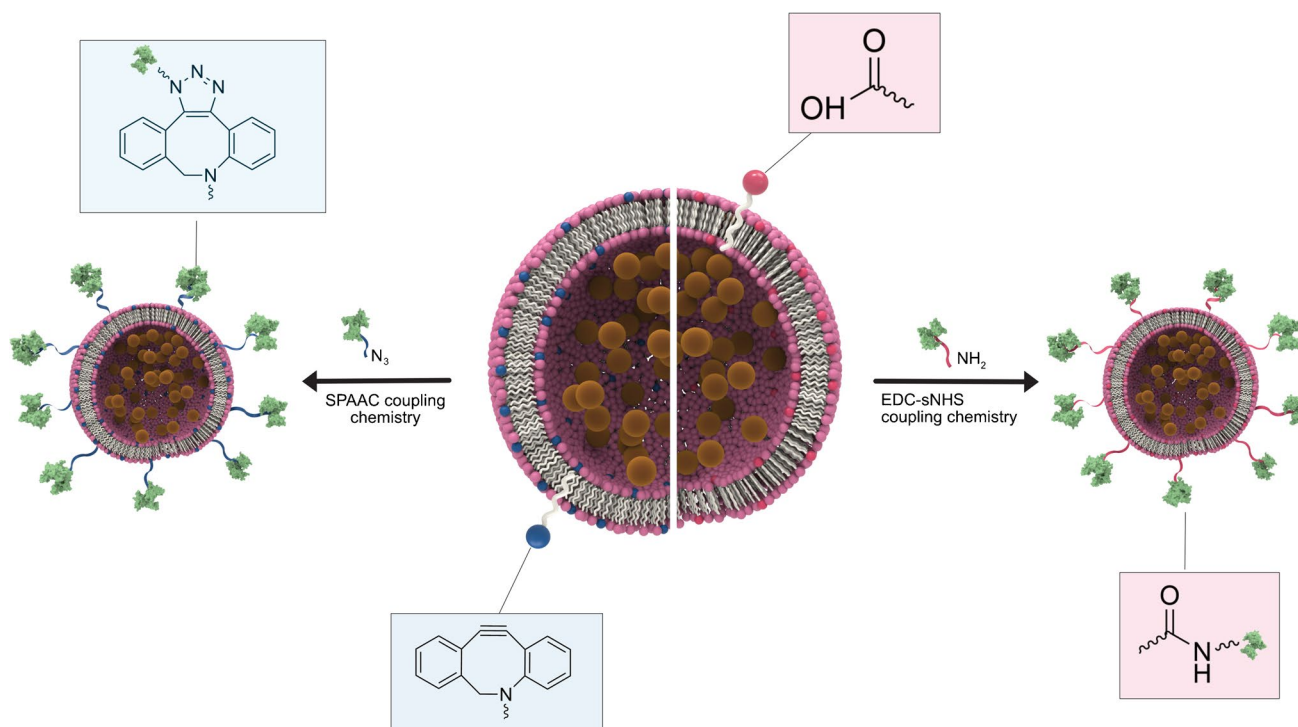


Fig. 1 Schematic representation of the two coupling methods (SPAAC click coupling chemistry and EDC-sNHS coupling chemistry) which were used for the functionalization of HRP encapsulating liposomes

encapsulating therapeutic proteins are modified with antibodies or other recognition elements for active targeting to a specific destination. The functionality of HRP can easily be tested via a simple TMB assay and is hence an ideal model protein. Thus, these HRP liposomes are compared to fluorescent SRB-encapsulating liposomes in a competitive assay for the detection of SARS-CoV-2 neutralizing antibodies. Beyond that, the simple surface modification of liposomes through the incorporation of functionalized lipids, together with the sensitive readout highlights the broad applicability of HRP liposomes as a versatile platform technology for a wide range of immunoassays.

Materials and methods

Chemicals and consumables

All chemicals and materials along with their suppliers are listed in the following. All chemicals were of analytical grade. *n*-Octyl- β -D-glucopyranoside (OG), 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES), sodium chloride (NaCl), potassium chloride (KCl), D-(+)-sucrose, sulfuric acid (H_2SO_4), and closure clips (Spectra/Por®) were purchased from Carl Roth (Karlsruhe, Germany); disodium hydrogen phosphate (Na_2HPO_4),

potassium dihydrogen phosphate (KH_2PO_4), sodium hydrogen carbonate (NaHCO_3), sodium hydroxide (NaOH), hydrochloric acid (HCl), ethanol, dimethyl sulfoxide (DMSO), 3-(13-azido-5,8,11-trioxa-2-azatridecanoate) cholesterol (cholesteryl-TEG-azide), and bovine serum albumin fraction V (BSA) from Merck (Darmstadt, Germany); cholesterol, Sephadex® G-50, Sephadex® G-100, TWEEN® 20, biotinylated bovine serum albumin, tris(hydroxymethyl)aminomethane (TRIS), horseradish peroxidase (HRP), 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate, human serum albumin (HSA), trehalose, and streptavidin from *Streptomyces avidinii* from Sigma-Aldrich (Steinheim, Germany); methanol, chloroform, nitric acid (65%), sulforhodamine B (SRB), Nunc MaxiSorp high-binding microplates, black or clear (96-well, flat bottom), polyclonal anti-RBD IgG antibody produced in rabbit (PA5-114451), and pierce protein concentrators (MWCO, 10 kDa) from Thermo Fisher Scientific (Waltham, USA); 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (DPPG), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-dibenzocyclooctyl (DPPE-DBCO), and the extrusion kit from Avanti Polar Lipids (Alabaster, USA); polycarbonate membranes with pore sizes of 0.2 μm , 0.4 μm , and 1.0 μm ($\varnothing$ 19 mm) and filter supports ($\varnothing$ 10 mm) from Whatman (Dassel, Germany); adhesive transparent foil for

microtiter plates, microcentrifuge tubes, glass chromatography columns, and falcon tubes from VWR Chemicals (Radnor, USA); black microtiter plates (96-well, flat-bottom) from Brand (Wertheim, Germany); Bio-One clear microtiter plates (96-well, flat bottom) from Greiner (Frickenhäusen, Germany); flat bottom, 96-well microtiter plate coated with polystreptavidin in black or white from Biomat snc (Rovereto, Italy); the dialysis membrane (Spectra/Por® Biotech CE with a MWCO of 1000 kDa or 12–14 kDa) from Spectrum Laboratories (Auckland, New Zealand); polymethyl methacrylate (PMMA) semi-micro cuvettes, and folded capillary zeta cells for DLS measurements from Malvern Panalytical (Malvern, UK); phosphorus standard for ICP measurements from Bernd Kraft (Den Haag, Netherlands); protein LoBind® tubes from Eppendorf® (Hamburg, Germany); pipette tips from Sarstedt (Nuembrecht, Germany); 2,3,5,6-tetrafluorophenyl 3-[2-[2-(2-azidoethoxy)ethoxy]ethoxy]propanoic acid (TFP-azide) from Quanta Bio-Design (Newark, USA); the biotinylation kit from Avidity (CO, USA); and pooled human serum samples (IRS41174, IRS45270, IRS46827, IRS56525 and IRS57906) from Innovative Research (Novi, USA). Human ACE2 receptor, ACE2-biotin, and SARS-CoV-2 RBD (Alpha B1.1.7) were provided by Microcoat Biotechnologie GmbH (Bernried, Germany).

Liposome preparation

Liposomes encapsulating the enzyme HRP were prepared using the thin-film hydration method. The lipid composition (approximately 60 μmol total lipids) usually contained 5 mol% cholesterol, 73 mol% DPPC, 18 mol% DPPG, and 4 mol% DMPE (non-clickable for HRP-content optimization) or DPPE-DBCO (clickable lipid, for all other synthesis). The lipid mixture was dissolved in 3 mL chloroform and 0.5 mL methanol. Next, the lipid composition was ultrasonicated for 1 min at 40 °C or 35 °C for non-clickable liposomes. The organic solvents were removed by a stepwise reduction of the pressure using a rotary evaporator (LAB-OROTA 4001 from Heidolph, Kelheim, Germany; speed, 180–210 rpm; 800 mbar for 2 min, 700 mbar for 2 min, 600 mbar for 2 min, 550 mbar for 5 min, 500 mbar for 5 min, 450 mbar for 5 min, 400 mbar for 5 min, 300 mbar for 2 min, 250 mbar for 5 min, and 200 mbar for at least 2 min). Afterwards, the lipids were completely dried under a nitrogen stream for 5–10 min until a thin and dry lipid film formed at the bottom of the flask. The thin lipid film was hydrated by addition of 1 mL of encapsulant (20–200 μM HRP, 310 mM sucrose or 11–20 w% of trehalose, 0.2 w/v% HSA in TRIS-HCl buffer, pH 7.2). The mixture was intensely vortexed for at least 2 min and sonicated at 40 °C (or 35 °C for non-clickable liposomes) until the lipid film detached from the glass flask. After letting the mixture rotate at 60 rpm and

800 mbar for 15 min, another 1 mL of encapsulant was added. The solution was vortexed for at least 2 min and ultrasonicated at 40 °C (or 35 °C for non-clickable liposomes) if the remaining lipids had not fully dissolved yet. The flask was transferred back to the rotary evaporator and rotated for 1 h to hydrate the lipid film (800 mbar and 60 rpm). The liposomes were extruded by repeated pushing of the solution through polycarbonate membranes with pore sizes of 1, 0.4, and 0.2 μm at 40 °C (from largest to smallest, 21 repetitions each). To separate free HRP from the liposomes, a size exclusion chromatography (SEC) was performed. Columns were filled with Sephadex G-100 in HS (column length of 20 cm and a diameter of 1.6 cm for liposomes 20 μM HRP and a column length of 50 cm with a diameter of 2.5 cm for liposomes encapsulating 50–200 μM HRP). The obtained fractions were grouped into high and medium fractions by turbidity. To separate remaining free HRP, a dialysis against HS with repeated buffer exchanges was done at 4 °C (Spectra/Por, MWCO, 1000 kDa). The liposomes were stored at 4 °C.

Liposomes encapsulating the fluorescent dye SRB were prepared as previously described using the reverse-phase evaporation method [43]. Briefly, lipids were dissolved in 3 mL chloroform and 0.5 mL methanol and ultrasonicated for 1 min at 60 °C. For the encapsulant (4.5 mL), SRB and NaCl were dissolved in 20 mM HEPES, pH 7.5. Two milliliters of the encapsulant was added to the lipid mixture and sonicated for 4 min at 60 °C. The organic solvents were evaporated by stepwise reduction of pressure at 60 °C (900 mbar for 10 min 850 mbar for 5 min, 800 mbar for 5 min, 780 mbar for 20 min), with a rotary evaporator. Before and after the second addition of 2 mL encapsulant, the solution was vortexed for 1 min. The residual organic solvent was removed at 60 °C by reduction of pressure (750 mbar for 20 min, 600 mbar for 5 min, 500 mbar for 5 min, 400 mbar for 20 min). The liposomes were extruded by repeated pushing of the solution through polycarbonate membranes with pore sizes of 1, 0.4, and 0.2 μm at 60 °C (from largest to smallest, 21 repetitions each). Size exclusion chromatography with a column filled with Sephadex G-50 in HS (column length of 20 cm and a diameter of 1.6 cm) was used for removal of the excess encapsulant, followed by dialysis at room temperature (RT) in a dialysis membrane Spectra/Por 4 (MWCO, 12–14 kDa) against HS buffer with repeated buffer exchanges. The liposomes were stored at 4 °C.

Liposome characterization

The total lipid concentration of liposomes was determined using ICP-OES measurements with a Spectroblue inductively coupled plasma optical emission spectrometer (SPECTRO Analytical Instruments GmbH, Kleve,

Germany). Therefore, the phosphorus concentration in the sample was measured at a wavelength of $\lambda = 177.495$ nm. The spectrometer was calibrated using a phosphorus standard solution with concentrations between 0 and 100 μM in 0.5 M HNO_3 . For every measurement, a recalibration was done using the 0 μM and 100 μM phosphorus dilutions. Liposome samples were diluted in 0.5 M HNO_3 before measuring the phosphorus content. Thereafter, the total lipid concentration (tL) was determined using the phospholipid concentration previously determined via ICP-OES, the liposome dilution, and the percentage of phospholipids in the liposomes.

The hydrodynamic diameter and the ζ -potential of the liposomes were determined with dynamic light scattering (DLS) measurements using the Malvern Zetasizer Nano-ZS (Malvern Panalytical, Malvern, UK). Therefore, liposomes were diluted to a concentration of 50 or 25 μM tL (unmodified or modified liposomes) in the outer liposome buffer (HS buffer; dispersant refractive index: $n_D^{20} = 1.33$; dielectric constant: $\epsilon = 78.5$; viscosity: $\eta = 0.9076$ cP). The size measurement was performed in PMMA semi-micro cuvettes (Brand, Wertheim, Germany) at 25 °C. Three measurements with 13 single measurements each were performed. The ζ -potential was measured in folded capillary zeta cells (Malvern Panalytical, Malvern, UK) after equilibration at 25 °C for 60 s. Four measurement runs were performed with each 30 single measurements.

For the maximum and initial absorbance measurements of HRP liposomes, they were diluted to different concentrations depending on their HRP content (between 0.1 and 2 μM tL, 0.2 μM tL for 100 μM HRP liposomes) in HS with or without 30 mM OG. After waiting 10–15 min for the liposomes to be lysed by OG, 100 μL TMB substrate was added to samples. The reaction was stopped after 5 min with 50 μL 1 M H_2SO_4 and the absorbance was measured at a wavelength of 450 nm ($n = 3$) using the BioTek SYNERGY neo2 multi-mode reader (Agilent Technologies, Santa Clara, USA).

For the maximum and initial fluorescence determination of SRB liposomes, they were diluted to 5 μM tL in HS with (max fluorescence of lysed liposomes) or without 30 mM OG (initial fluorescence of unlysed liposomes), each in triplicates. After waiting 20 min for the liposomes to be lysed by OG, the fluorescence was measured using the BioTek SYNERGY neo2 multi-mode reader (Agilent Technologies, Santa Clara, USA) ($\lambda_{\text{ex}} = 560$ nm and $\lambda_{\text{em}} = 585$ nm, bandwidth (BW) = 10 nm, gain 100, $n = 3$, measurement from the top).

The so-called initial fluorescence/absorbance was calculated as the ratio of the fluorescence intensities or absorbance of unlysed and lysed liposomes. The values were averaged and the blank (buffer) signal was subtracted.

Surface modification via SPAAC

For the click reaction, liposomes with the clickable lipid DBCO PE were prepared. The protein of choice was functionalized with a corresponding azide moiety via an azido-dPEG₄-TFP ester. Therefore, the azido-dPEG₄-TFP ester was dissolved in 20 v% DMSO and 80 v% HS buffer. Azide was added in excess to the respective protein (streptavidin, 1:25; RBD, 1:50; protein to azide ratio). Samples were incubated for 2 h at RT and 300 rpm. The modified protein sample was purified using centrifuge filters at 12,000 RCF for 5 min (MWCO, 10 kDa) with five subsequent washing steps with 400 μL HS buffer. Protein concentrations were determined in quadruplets using the NanoPhotometer® NP80 (Implen, Westlake Village, USA).

The azide-modified protein was added to liposomes containing 4 mol% DBCO PE. The amount needed for the specific surface coverage, e.g., 0.2 mol%, was calculated using the total lipid concentration, which was previously determined via ICP-OES, and the protein concentration. The mixture was typically incubated for 24 h at RT and 300 rpm although during the optimization, different temperatures and times were tested. Uncoupled protein was removed via dialysis in a dialysis membrane from Spectra/Por (MWCO, 1000 kDa) against HS buffer at 4 °C with repeated buffer exchange. Protein-modified liposomes were stored at 4 °C.

Biotinylation of RBD

Biotinylation of RBD via its Avi-tag was performed using the lyophilized BirA500RT biotinylation kit from Avidity according to the manufacturer's instruction. Briefly, supermix, RBD, BirA, and water were incubated for 4 h at RT and 300 rpm. Excess reagents were removed using centrifuge filters at 12,000 RCF for 5 min (MWCO, 10 kDa) with five subsequent washing steps with 400 μL HS buffer. Protein concentrations were determined in quadruplets using a NanoDrop (Thermo Fisher Scientific, Waltham, USA).

The biotinylated RBD was attached to HRP liposomes, which were modified with 1 mol% streptavidin via SPAAC.

Determination of enzyme activity

To test the activity of enzyme samples, the solutions were diluted in HS to a concentration of 30 or 40 pM HRP (30 pM for HRP in different buffer solutions and 40 pM for HRP combined with different amounts of HSA). The samples were pipetted into a clear microtiter plate (100 μL , $n = 4$). One hundred microliters of TMB per well were added. The reaction was stopped after 5 min with 1 M H_2SO_4 (50 μL). The absorbance was measured with the BioTek Synergy

Neo2 Multimode Microplate Reader at 25 °C and a wavelength of $\lambda = 450$ nm three consecutive times. The values were averaged, and the blank (buffer) signal was subtracted.

Heterogenous binding assay

BSA-biotin (2 $\mu\text{g/mL}$ in phosphate-buffered saline (PBS) consisting of 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , 137 mM NaCl, and 2.7 mM KCl, pH 7.4; 100 μL) was immobilized in a high-binding microtiter plate (Thermo Fisher) overnight at 4 °C. The solution was removed and the plate was blocked with 1 w/v% BSA in PBS-T (PBS with 1 w/v% TWEEN® 20, 150 μL) for 1 h at RT and 300 rpm. Afterwards, the plate was washed twice with PBS-T (150 μL) and three times with HS buffer (150 μL). Afterward, the liposomes (100 μL , 10 μM tL in HS for SRB liposomes and 5 μM tL in HS for HRP liposomes) were added to the respective wells and incubated for 3 h at RT (SRB liposomes) or at 4 °C (HRP liposomes) and 300 rpm. The plate was washed three times with HS (150 μL) at RT.

SRB liposomes: The plate was incubated for 20 min at RT and 300 rpm with 30 mM OG in double-distilled water (100 μL). The fluorescence was measured with the BioTek Synergy Neo2 Multimode Microplate Reader at RT three consecutive times ($\lambda_{\text{Ex}} = 560$ nm and $\lambda_{\text{Em}} = 585$ nm, bandwidth 10, gain 100 and 150). The values were averaged, and the blank (buffer) signal was subtracted.

HRP liposomes: After waiting for 10 min for the liposomes to be lysed by OG (30 mM OG in double-distilled water (100 μL)), 100 μL of TMB per well was added. The reaction was stopped after 5 min with 1 M H_2SO_4 (50 μL per well). The absorbance was measured with the BioTek Synergy Neo2 Multimode Microplate Reader at 25 °C and a wavelength of $\lambda = 450$ nm three consecutive times. The values were averaged, and the blank (buffer) signal was subtracted.

Neutralization assay and serum screening

A streptavidin microtiter plate was coated with ACE2-biotin (1 $\mu\text{g/mL}$ in PBS, 100 μL) for 1 h at RT and 300 rpm. Meanwhile, RBD liposomes were diluted to 1 or 2 μM tL, mixed with antibody or serum dilutions in HS buffer, and incubated for 1 h at 30 °C. The ACE2-biotin solution was removed and the plate was washed twice with PBS-T (150 μL) and three times with HS buffer (150 μL). Afterwards, the liposome-antibody/serum mixtures were added to the plate (100 μL) and incubated for 2 h at RT (SRB liposomes) or 3 h at 4 °C (HRP liposomes). The plate was washed three times with HS (150 μL) at RT to remove unbound liposomes. Immobilized liposomes were lysed by incubation with 30 mM OG in double-distilled water (100 μL , 10 min for HRP liposomes or 20 min for SRB liposomes).

SRB liposomes: The fluorescence was measured with the BioTek Synergy Neo2 Multimode Microplate Reader at RT three consecutive times ($\lambda_{\text{Ex}} = 560$ nm and $\lambda_{\text{Em}} = 585$ nm, bandwidth 10, gain 100 and 150). The values were averaged, and the blank (buffer) signal was subtracted.

HRP liposomes: After liposome lysis, 100 μL of TMB per well was added. The reaction was stopped after 5 min with 1 M H_2SO_4 (50 μL per well). The absorbance was measured with the BioTek Synergy Neo2 Multimode Microplate Reader at 25 °C and a wavelength of $\lambda = 450$ nm three consecutive times. The values were averaged, and the blank (buffer) signal was subtracted.

Results and discussion

The coupling of proteins to liposomes has been standardly done applying EDC-sNHS chemistry when using fluorescent liposomes [44–47]. Here, liposomes entrapping 10 μM HRP were prepared using the thin-film hydration method, resulting in stable liposomes and providing a quantitative photometric readout upon detergent-based lysis of the liposomes (Table SI 1). However, the entrapped HRP was found to rapidly lose its enzymatic activity upon streptavidin modification of the liposomal surface using EDC-sNHS chemistry (Figure SI 1). Enzyme activity measurements after each reaction step, including addition of EDC-sNHS, streptavidin, lysine HCl, and dialysis, revealed a deactivation of the enzyme after each single step. Thus, we hypothesize that EDC or sulfo-NHS can penetrate the lipid bilayer and react with encapsulated HRP molecules either by crosslinking the HRP molecules themselves or by attaching the enzymes to the carboxyl groups of the inner liposomal layer. Such reactions could induce progressive changes in the enzyme's 3D structure, crosslinking, or inadequate mobility during the substrate conversion, as also suggested by Kazenwadel et al. [48], ultimately leading to a loss of catalytic activity over time. Further studies are necessary to determine the exact cause of the deactivation but it was not focus of this study since it could be circumvented by readily accessible strategies. Therefore, a copper-free click chemistry strategy was investigated as a promising alternative with the goal of obtaining a general coupling protocol that is as efficient as the established EDC-sNHS chemistry but without affecting entrapped enzyme molecules.

Based on many reports in literature, SPAAC coupling was chosen as an appropriate alternative to EDC-sNHS chemistry. In the case of liposomes, either the alkyne or the azide moiety can be incorporated into the lipid bilayer through respective phospholipids. The protein of interest can be readily modified with the complementary click-reactive group, allowing its covalent coupling to the liposome surface. Both types of liposomes were hence prepared incorporating

cholesteryl-TEG-azide or DBCO DPPE as clickable lipids, respectively. The resulting liposomes were characterized using ICP-OES, DLS, ζ -potential, and absorbance measurements and all parameters were within the expected range for liposomes prepared by standard synthesis procedures (Fig. 2B). In the case of azide-functionalized liposomes, the long-term storage exhibited a continuous and drastic loss of activity of the entrapped HRP, where 93% of activity was lost after 4 weeks storage (Fig. 2A). It is assumed that the azide moieties of the lipids directed toward the inner volume of the liposomes caused this deactivation [49, 50]. Azide is known to coordinate to the prosthetic heme iron atom of enzymes and thereby inhibits the HRP-TMB- H_2O_2 reaction by blocking the H_2O_2 binding site and the following oxidation of TMB; specifically, azidyl radicals may be added to one of the meso positions of the prosthetic group [50]. Furthermore, the azide acts as an inhibitor of the HRP-TMB reaction as it inhibits the hydrogen donor system between HRP and hydrogen peroxide [49].

In the case of liposomes incorporating the clickable lipid DBCO PE, the entrapped HRP remarkably retained its full activity for 12 months (Fig. 2A). Furthermore, even under prolonged incubation periods at RT for up to 96 h and various buffer compositions, no HRP activity was lost providing flexible coupling and assay conditions (Figure SI 2).

To enhance the assay sensitivity, the entrapped HRP content was optimized by preparing liposomes containing 20, 50, and 100 μM HRP. Not surprisingly, higher HRP concentrations led to better performing liposomes, i.e., steeper dose-response curves and LODs improved from 14 to 2.8 nM (Figure SI 3). Considering the HRP concentrations recommended by suppliers, the highest encapsulant concentration obtainable is 230 μM , suggesting that further

performance increases are feasible but were not studied here. It is common practice that bulk proteins or sugars are added for long-term storage of enzymes [51–54]; hence, entrapped HRP was previously stabilized via 2 mg/mL HSA and 310 mM sucrose in TRIS-HCl buffer (pH 7.2). Due to insufficient stability after the coupling process (Fig. 3A), the encapsulant composition was further optimized. The combination of 100 μM HRP with various HSA concentrations in TRIS-HCl buffer again indicated high stability of the enzyme and HSA was therefore chosen as an encapsulant additive to be entrapped in the liposomes alongside the enzyme (Figure SI 4). It was found that higher HSA concentrations provided no further benefit beyond the stabilization observed for the already used HSA content. Higher contents may be of value when other enzymes are entrapped within the liposomes that are overall less stable than HRP.

More importantly, the long-term stability of the liposomes was also studied after coupling via SPAAC with 0.2 mol% streptavidin-azide. The coupling solution of the control sample contained no streptavidin-azide but was treated like the other samples to ensure that possible changes in the enzyme activity were a result of the coupled protein but not of the coupling procedure. This sample is referred to as 0 mol% streptavidin sample. Successful coupling was confirmed by DLS and ζ -potential measurements as well as binding assays where BSA-biotin was immobilized in a microtiter plate (Figure SI 5, Table SI 2). Liposomes modified with 0 mol% (control) or 0.2 mol% streptavidin were allowed to bind to the immobilized BSA-biotin to prove successful modification and correct orientation. After incubation, unbound liposomes were washed away, and remaining liposomes were quantified after lysis with a detergent. Liposomes entrapping 100 μM HRP in TRIS-HCl buffer with HSA (2 mg/mL)

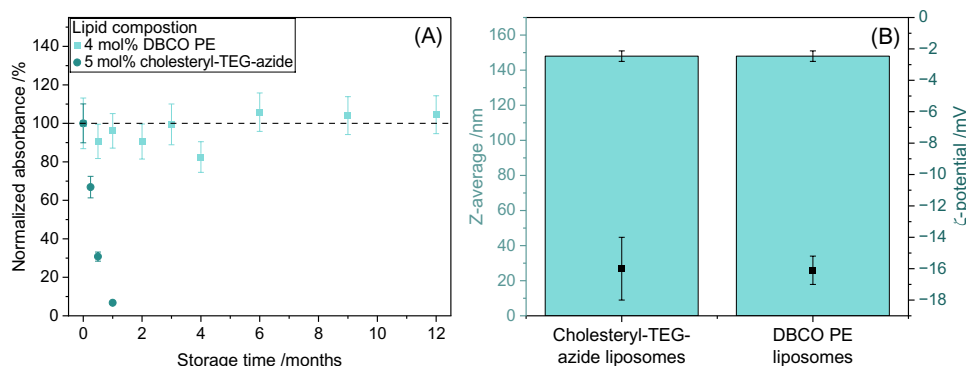


Fig. 2 Characterization of liposomes containing clickable lipids. **(A)** Normalized absorbance of 10 μM HRP liposomes (2 or 2.6 μM tL) as a measure of enzyme activity, containing 4 mol% of DBCO PE or 5 mol% cholesteryl-TEG-azide in their lipid bilayer and 310 mM sucrose and 2 mg/mL HSA in their encapsulant in addition to the encapsulated HRP. Maximum absorbance was achieved by lysis of the liposomes with 30 mM OG in HS buffer (10 min), followed by

TMB/HRP reaction (5 min). The absorbance was measured in triplicates at RT three consecutive times ($\lambda = 450$ nm, $T = 25$ °C, $n = 3$) and adjusted for the buffer control. **(B)** Diameter (z -average) and surface charge (ζ -potential) of the clickable 10 μM HRP liposomes DBCO PE or cholesteryl-TEG-azide liposomes (c(tL) = 50 or 77 μM tL in HS buffer, $T = 25$ °C, $n = 3$)

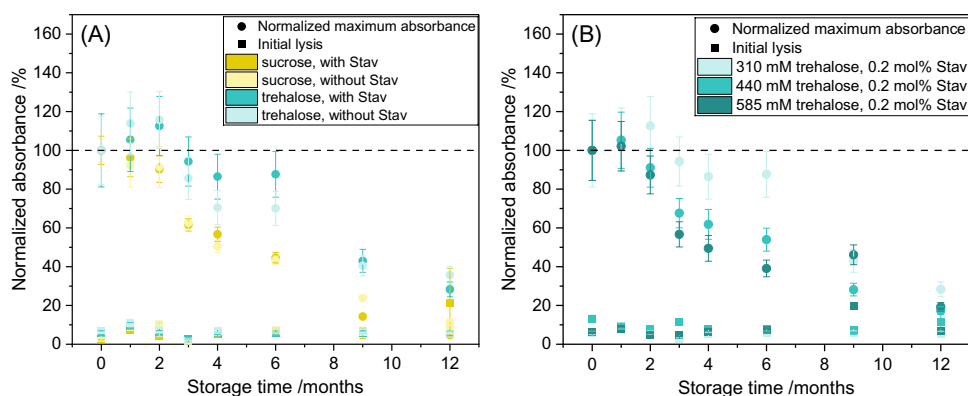


Fig. 3 Optimization of encapsulant composition to ensure enzyme stability. Normalized absorbance of 100 μ M HRP liposomes after coupling to 0 or 0.2 mol% of streptavidin-azide and storage at 4 °C of liposomes containing (A) 310 mM sucrose or trehalose or (B) 310 to 585 mM trehalose: Maximum absorbance was achieved by lysis with

and stabilized by the addition of either 310 mM sucrose or trehalose were investigated. These sugars and their concentration were chosen based on prior experience. Trehalose was found to maintain HRP activity far better than sucrose throughout the entire storage study which might be caused by the larger hydrated volume generated by trehalose than by sucrose (Fig. 3A) [53]. Furthermore, the difference between coupled and control liposomes was insignificant. However, storage for longer than 6 months was surprisingly not possible as the signal dropped to 40% activity. Even higher trehalose concentrations (up to 585 mM) would not improve this drop in activity. Hence, the optimal buffer composition to ensure stable liposomes after modification for up to six months is 2 mg/mL HSA and 310 mM trehalose dissolved in TRIS-HCl alongside the required HRP content. Further studies are needed to better protect HRP activity if storage for longer than 6 months is desired.

Optimization of coupling conditions was carried out using liposomes that encapsulate the fluorescent dye SRB to determine the most effective modification conditions before applying those to HRP liposomes to understand their influence on the entrapped enzyme. This allowed the direct comparison to the established EDC-sNHS chemistry and avoided a convolution of possible entrapped enzyme effects from mere coupling chemistry efficiency. Thus SPAAC was applied to 10 mM SRB-encapsulating liposomes and streptavidin as a model protein. Specifically, streptavidin was modified with an azide moiety (ratio 1:25) and incubated with liposomes containing DBCO PE. Parameters varied including coupling temperature (4 °C, 15 °C, 22 °C), incubation time (from 18 to 96 h), and streptavidin content (0.2–1.5 mol%). Successful modification with streptavidin was confirmed via DLS and ζ -potential measurements (Figure SI 6). The

30 mM OG and subsequent TMB/HRP reaction (5 min, stopped with 1 M sulfuric acid). The absorbance was measured in triplicates at RT three consecutive times ($\lambda=450$ nm, $T=25$ °C, $n=3$) and adjusted for the buffer control. Initial lysis was calculated by dividing the signal from unlysed liposomes by that from lysed liposomes

coupling efficiency of the click reaction was studied in a heterogenous binding assay with BSA-biotin immobilized in a microtiter plate. Here, higher signals indicate more binding and therefore a presumably denser surface coverage with streptavidin (Fig. 4). The temperature during the coupling reaction was found to have no effect on the click reaction, as all samples showed a similar binding efficiency independent of the coupling temperature (Fig. 4A). In contrast, the incubation time had a significant effect at all temperatures tested (Fig. 4A), which was further confirmed in a more in-depth analysis at RT (Fig. 4B). These findings are in good accordance with previous findings from Blenke et al. who found that the SPAAC coupling of liposomes to 5'-TAMRA-azide, a commercially available dye, is not completed within 24 h at liposome concentrations similar to those used here [30]. Based on their results, coupling times can be shortened in the future with overall higher liposome concentrations. For the comparison to EDC-sNHS-modified SRB liposomes [56], an incubation of 24 h at RT was chosen for the SPAAC reaction (Fig. 4C), and streptavidin concentrations varied between 0.2 and 1.5 mol%. Previous studies have shown that streptavidin concentrations between 1 and 1.5 mol% provided similar and optimal results in the binding assay, which is why no higher streptavidin concentrations were investigated here [56]. Interestingly, at the lowest protein concentration, the SPAAC reaction produced signals which were twice as high as those of the EDC-sNHS-coupled liposomes. In contrast, at the highest concentration, SPAAC coupling reached only 67% of the signal achieved with EDC-sNHS chemistry. This is most likely a concentration effect which is well known to affect the SPAAC reaction [30, 57]. Due to the dilution effect of the added protein solution, the liposome concentration,

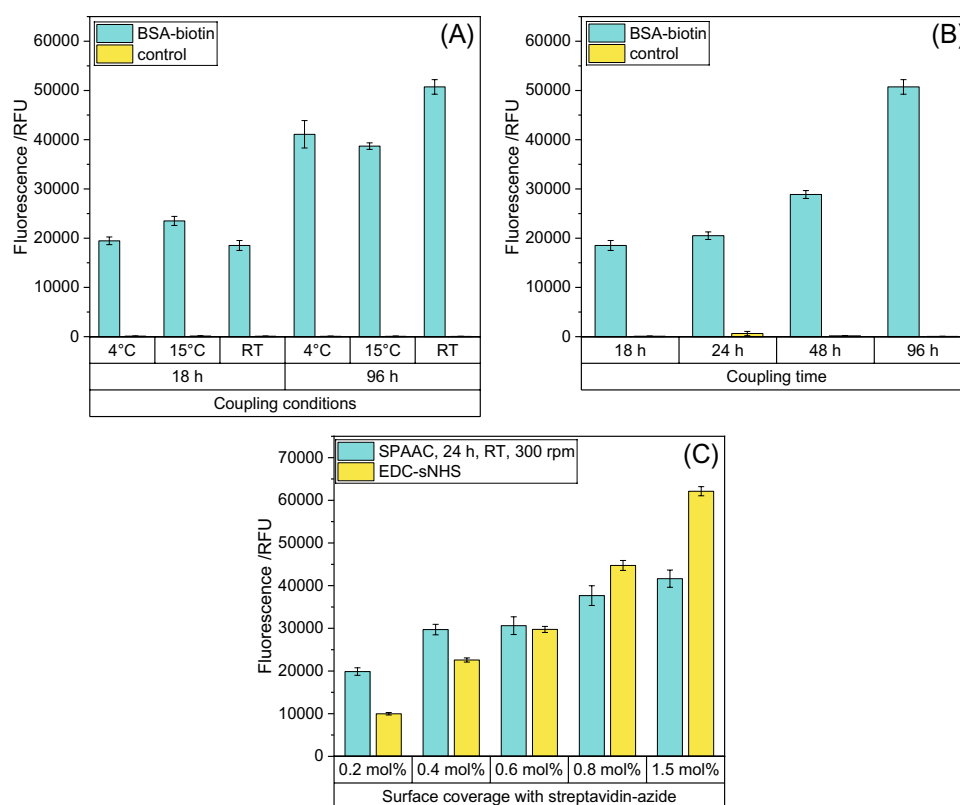


Fig. 4 Optimization of SPAAC conditions and comparison to EDC-sNHS chemistry using binding assays. Streptavidin liposomes (10 μ M tL, 100 μ L per well, liposome-encapsulant: 10 mM SRB) were immobilized in a black high-binding microtiter plate coated with BSA-biotin (2 μ g/mL, 100 μ L) and blocked with 1 w/v% BSA in PBS-T. Before addition of liposomes for 3 h at RT and 300 rpm the plate was washed with PBS-T (2 $\times$ 150 μ L) and HS (3 $\times$ 150 μ L). Liposomes were lysed by addition of 30 mM OG in double-distilled water (100

μ L). The fluorescence was measured in triplicates using $\lambda_{\text{ex}}=560(10)$ nm and $\lambda_{\text{em}}=585(10)$ nm, gain 150. $n=3$. (A) Effect of different incubation temperatures during coupling. (B) Coupling efficiency after 18, 24, 48, or 96 h of incubation at RT. (C) Comparison of coupling efficiencies between liposomes coupled via click chemistry (SPAAC) or via EDC-sNHS chemistry. Data of liposomes coupled via EDC-sNHS chemistry were previously published [55]

and therefore also the DBCO concentration, is about twice as high in the sample with 0.2 mol% streptavidin (3 mM tL) as in the one with 1.5 mol% streptavidin (1.2 mM tL). If desired, higher protein surface coverages via the SPAAC reaction can be obtained by increasing the incubation time from 24 to 96 h in the future. Overall, it can be concluded that the SPAAC reaction can be an efficient way of protein modification of liposomes performing similarly well as the widely employed EDC-sNHS chemistry. Hence, other click chemistry strategies, such as those with copper catalysts, are not needed [39, 41, 58].

The optimized liposome synthesis and coupling protocols were then applied toward the detection of neutralizing antibodies against SARS-CoV-2 as demonstrated by Streif et al. where the liposome surface is modified with the receptor binding domain (RBD) of SARS-CoV-2 [59]. In this assay format, neutralizing antibodies present in serum prevent the binding of RBD-modified liposomes to immobilized human ACE2, the receptor responsible for SARS-CoV-2 entry into host cells. SRB-entrapping liposomes relying on

fluorescence detection to detect SARS-CoV-2 neutralizing antibodies were shown to correlate very well with a pseudovirus neutralization test with a Spearman r of 0.75 [59].

Since fluorescence detection is not available in many routine labs and similarly does not lend itself well for point-of-care, the adaptation to a photometric readout while maintaining the simple assay format and its high sensitivity is highly desirable, making it an ideal application for the HRP-entrapping liposomes developed here. Therefore, liposomes entrapping 100 μ M HRP were coupled using SPAAC to varying concentrations of azide-modified RBD (0–1 mol%) and evaluated through their binding to immobilized ACE2 (Fig. 5A). As expected, increasing RBD concentrations between 0.05 and 0.5 mol% lead to higher signals and thus more efficient binding. However, further increases led to a drop in binding ability, which is assumed to be caused by a crowding of protein molecules on the liposome surface resulting in steric hindrance in their binding to ACE2, as RBD is coupled in a non-directed manner to the liposomal surface. It should be noted that absorbance values above 1

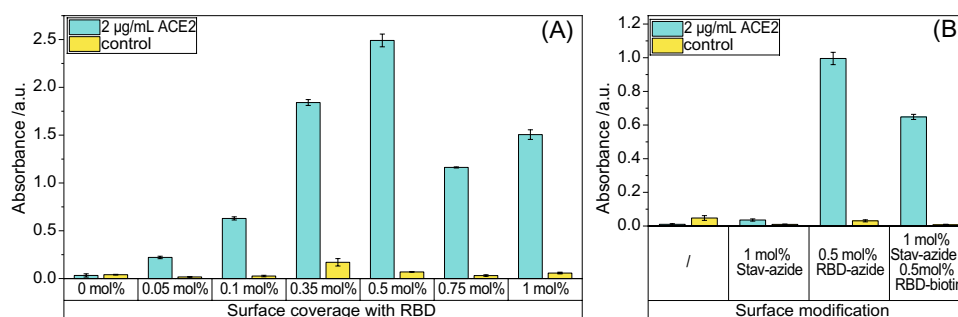


Fig. 5 (A) Optimization of liposome surface coverage with RBD: Liposomes were modified with 0.05–1 mol% RBD-azide via SPAAC and afterward tested in a binding assay. Absorbance values above 1 were only considered qualitatively. (B) Comparison of liposomes which were either first modified with streptavidin-azide via SPAAC and afterwards further modified with RBD-biotin via streptavidin-biotin interaction or directly modified with RBD-azide via SPAAC. For the respective binding assays, liposomes (10 µM tL, 100 µL per well, liposome-encapsulant: 100 µM HRP, 2 mg/mL HSA, 310 mM

trehalose in TRIS-HCl) were immobilized in a clear high-binding microtiter plate coated with ACE2 (2 µg/mL, 100 µL) and blocked with 1 w/v% BSA in PBS-T. Before adding liposomes for 3 h at 4 °C and 300 rpm the plate was washed with PBS-T (2× 150 µL) and HS (3× 150 µL). Liposomes were lysed by addition of 30 mM OG in double-distilled water (100 µL). The absorbance ($\lambda=450$ nm, buffer-adjusted) of TMB/H₂O₂ oxidation product (5 min, 100 µL TMB, 50 µL sulfuric acid) was measured in triplicates using $\lambda=450$ nm. ($n=3$)

were only considered qualitatively. Still, among the concentrations tested, 0.5 mol% RBD provided the best results. To avoid the modification of RBD with azide, it was also investigated to couple biotinylated RBD to streptavidin-modified liposomes (Fig. 5B). Therefore, liposomes were first modified with 1 mol% of streptavidin via the optimized SPAAC reaction and then incubated with 0.5 mol% of biotinylated RBD. RBD was biotinylated through its C-terminal Avi-tag using a commercial BirA biotin-protein ligase kit. Their performance in an ACE2 binding assay was compared to liposomes coupled directly to RBD-azide. Interestingly, the direct coupling approach resulted in 54% higher signals than the approach using streptavidin liposomes along with the biotinylated RBD. We assume that fewer biotinylated RBD molecules were bound to the liposomes due to the favorable presence of 4 mol% DBCO PE vs. only 1 mol% streptavidin per liposome. However, since many proteins are available with biotin tags, this latter approach is of high value and can be further optimized in the future, in particular for the site-directed immobilization of proteins on the liposome surface.

To further increase the sensitivity, 200 µM HRP liposomes were prepared. Subsequently, 100 and 200 µM HRP-entrapping liposomes modified via SPAAC with 0.5 mol% RBD-azide were compared to the 10 mM SRB-entrapping liposomes. First, the lowest concentration of total lipid detectable of all liposomes was determined and found to be 6.9 nM for the SRB liposomes, and 1.1 nM and 0.48 nM for the 100 µM and 200 µM HRP liposomes, respectively (Fig. 6A). This demonstrates that HRP liposomes relying on photometric detection can outperform SRB liposomes with fluorescence detection. Secondly, binding of the liposomes to immobilized ACE2 was investigated (Fig. 6B). Also here, the LODs obtained were very similar with 67 nM for the

SRB liposomes, and 51 nM or 22 nM for the 100 µM or 200 µM HRP liposomes, respectively, verifying that HRP liposomes deliver the same sensitivity as fluorescent SRB liposomes. It should be noted that, as expected, 200 µM HRP liposomes further lowered the LOD by a factor of 2 in comparison to the liposomes entrapping 100 µM HRP, suggesting that even higher entrapped HRP concentrations and lower LODs are feasible as discussed above.

The different HRP and SRB liposomes were then used for the detection of a commercially available neutralizing antibody (PA5-114451) as proof of principle. The polyclonal PA5-114451 antibody was previously found to fully block binding of SRB liposomes modified with RBD via EDC-sNHS to the immobilized ACE2 receptor. Its binding site is within the receptor binding motif and it can therefore outcompete RBD's binding to ACE2 (class 1 and 2 antibodies) [59, 60]. Thus, higher antibody concentrations will lead to less liposome binding and hence lower signals in the binding assay. This signal-off strategy was already optimized for SRB-encapsulating liposomes and applied here for all liposomes [59]. A schematic representation of the assay principle can be seen in Figure SI 7. Indeed, HRP liposomes showed a similar ability to detect the neutralizing antibody (Fig. 7), with an EC_{50} of 0.042 ± 0.008 µg/mL for SRB liposomes and an EC_{50} of 0.196 ± 0.008 µg/mL for HRP liposomes when using 2 µM tL. To lower the EC_{50} value obtained by HRP liposomes, their concentration was dropped, and hence also the amount of RBD that needs to be neutralized, while increasing the TMB reaction time to compensate for otherwise lower signals. Indeed, reducing the liposome concentration from 2 µM to 1 µM tL and prolonging the reaction time from 10 to 30 min, led to an EC_{50} of 0.043 ± 0.004 µg/mL just as the one obtained by the SRB

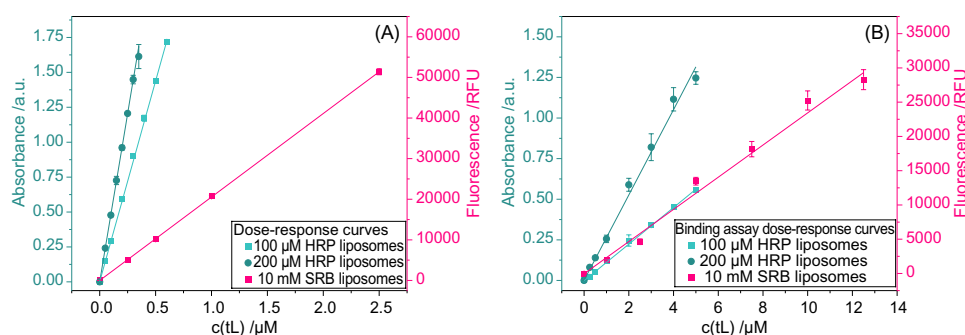


Fig. 6 Sensitivity of 100 or 200 μM HRP or 10 mM SRB-encapsulating liposomes coupled with 0.5 mol% RBD-azide via SPAAC. (A) Dose-response curve of liposomes encapsulating 100 or 200 μM HRP modified with 0.5 mol%-RBD: 100 μM HRP: $R^2=0.99987$; corresponding LOD and LOQ of 1.1 and 3.5 nM, respectively. 200 μM HRP: $R^2=0.99997$; corresponding LOD and LOQ of 0.48 and 1.5 nM, respectively. Dose-response curve of SRB liposomes modified with 0.5 mol%-RBD: $R^2=0.99992$; corresponding LOD

and LOQ of 6.9 and 21 nM, respectively. (B) Dose-response curve of liposomes encapsulating 100 or 200 μM HRP modified with 0.5 mol%-RBD in a binding assay: 100 μM HRP: $R^2=0.99885$; corresponding LOD and LOQ of 51 and 150 nM, respectively. 200 μM HRP: $R^2=0.99388$; corresponding LOD and LOQ of 22 and 66 nM, respectively. Dose-response curve of SRB liposomes modified with 0.5 mol%-RBD in a binding assay: $R^2=0.99992$; corresponding LOD and LOQ of 67 and 200 nM, respectively

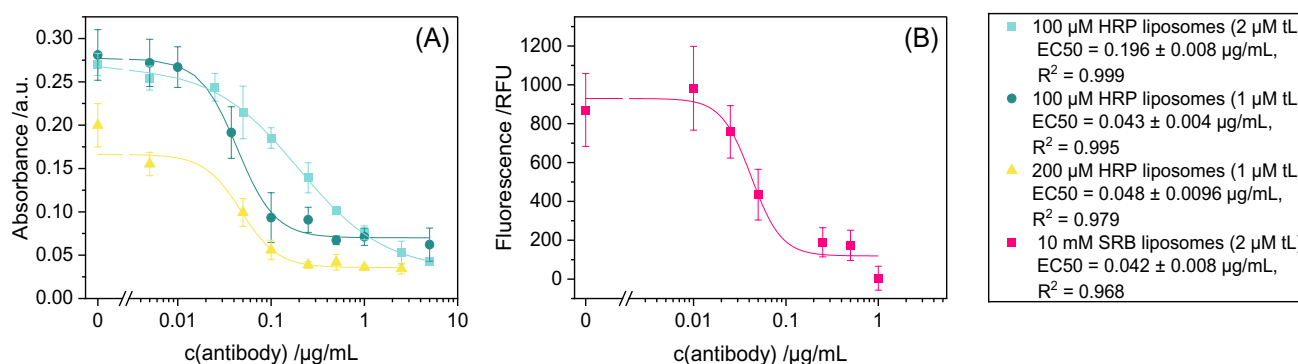


Fig. 7 Neutralization curves of 0.5 mol% RBD liposomes encapsulating 100 or 200 μM HRP (A) or 10 mM SRB (B). 0.5 mol% RBD liposomes were incubated with neutralizing antibody PA5-114451 (0–1 $\mu\text{g/mL}$) for 1 h at 30 $^{\circ}\text{C}$ and 300 rpm. Afterwards, the mixture was added to an ACE2-biotin-polystyrene-coated plate (1 $\mu\text{g/mL}$)

ACE2-biotin) and incubated for 3 h at 4 $^{\circ}\text{C}$ (HRP liposomes) or 2 h at RT (SRB liposomes) and 300 rpm. After washing and lysis of immobilized liposomes, the absorbance or fluorescence was measured in triplicates. Curves were fitted using Origin's logistic function

liposomes. It is important to note that liposomes entrapping higher HRP concentrations (e.g., 200 μM) may give higher overall signals, but obviously do not provide different EC_{50} values ($0.048 \pm 0.0096 \mu\text{g/mL}$), as these are a characteristic of the antibodies tested and also depend on the total RBD content that needs to be neutralized, confirming the validity of the liposomal approach.

Finally, HRP liposomes were tested in the actual serum matrix, using five different pooled human neutralizing sera, and compared to the fluorescent SRB liposomes using previously optimized assay conditions [59]. The obtained EC_{50} values for the 200 μM HRP (Fig. 8A) and the 10 mM SRB liposomes (Fig. 8B) correlated strongly and yielded a Pearson's r of 0.987. These results show that absorbance-based enzyme encapsulating liposomes represent a reliable alternative to liposomes which encapsulate fluorescent dyes.

Conclusion

Conjugation of proteins and other biomolecules to the liposomal surface is well established. In the field of bioanalysis, most research is done using liposomes entrapping fluorescent dyes to take advantage of the highly sensitive detection strategy and the ease of separation between intact and lysed liposomes. However, routine labs and point-of-care diagnostics are generally not equipped with fluorescence detectors. Hence, the overall aim of this study was the development of liposomes with an absorbance-based readout that would achieve a similar sensitivity as those entrapping fluorescent dyes. While entrapment of enzymes such as HRP into liposomes is not new, it was quickly determined that their liposomal surface modification using standard

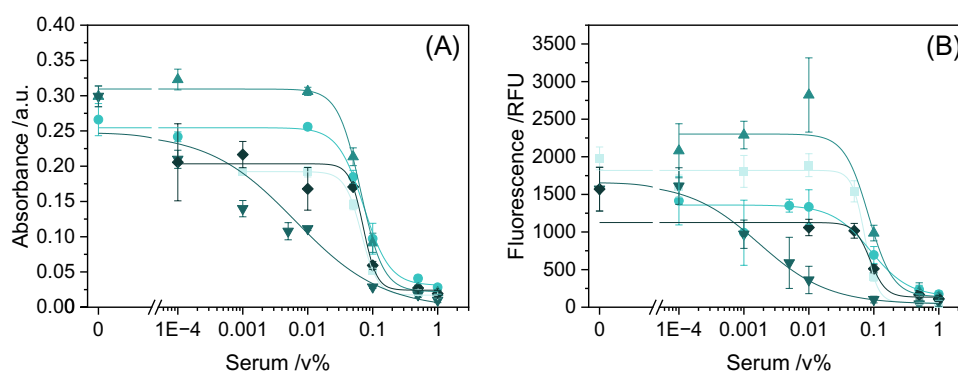


Fig. 8 Serum screening using (A) 200 μ M HRP encapsulating liposomes or (B) 10 mM SRB-encapsulating liposomes modified with 0.5 mol% RBD via SPAAC: 0.5 mol% RBD liposomes were mixed with different serum dilutions (0.0001 to 1 v% of serum) and incubated for 1 h at 30 °C and 300 rpm. Afterwards, the mixture was

added to an ACE2-biotin-coated polystyrene plate (1 μ g/mL ACE2-biotin) and incubated for 3 h at 4 °C (HRP liposomes) or for 2 h at RT (SRB liposomes) and 300 rpm. After washing and lysis of immobilized liposomes, the absorbance or fluorescence was measured in triplicates. Curves were fitted using Origin's logistic function

EDC-sulfo-NHS chemistry led to an inactivation of the entrapped HRP molecules. Therefore, the copper-free click reaction was studied and optimized using streptavidin as a model protein. The resulting HRP-entrapping liposomes modified with streptavidin at their outer surface performed as well as fluorescent liposomes. This was further confirmed in an actual bioassay for the detection of SARS-CoV-2 neutralizing antibodies using the SRB-encapsulating liposomes. Testing different neutralizing human sera, the results of HRP and SRB-encapsulating liposomes were found to be very similar (Pearson's r of 0.987).

The double signal amplification through the encapsulation of several enzymes and through the conversion of numerous substrate molecules suggests that liposome bioassays with an absorbance-based readout strategies can replace fluorescence-based ones in the future without a decrease in sensitivity. This facilitates the implementation of various bioassays for high-throughput screening and point-of-care technology, thereby simplifying both assay workflows and detection procedures. SPAAC-based modification offers a flexible and easily adaptable platform technology that can be tailored to the detection of various analytes of interest. SPAAC is particularly attractive for site-directed coupling strategies but requires the incorporation of suitable clickable reactive groups into the protein structure, for instance by using non-canonical amino acids. Altogether, this demonstrates the multitude of possible applications in the future.

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Author contribution Conceptualization: AJB, KH, and CD; performed experiments: CD; performed coupling of liposomes with streptavidin via EDC-sNHS: KH; writing (original draft): CD; writing (review and editing): AJB and KH. All authors have read and agreed to the published version of the manuscript.

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Data availability The data that support the findings of this study are available from the authors upon reasonable request.

Declarations

Conflict of interest The authors declare no competing financial interest. Antje J. Baemner is editor of *Analytical and Bioanalytical Chemistry* but was not involved in the peer review of this manuscript. The remaining authors declare no competing interests.

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