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Surface-functionalized fluorescent silica nanoparticles for the detection of ATP†

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The design of two-dyed fluorescent silica nanoparticles for ATP detection is presented. The indicator dye possesses a dipicolylamine (DPA) unit complexed with Zn(II) as a receptor function for ATP while a rhodamine derivative is used as the reference dye. The nanoparticles were fully characterized regarding analytical performance, morphology and cytocompatibility.

The development of new analytical systems for small biomolecules of significant medical interest has increased over the past decades. Within this scope, one of the mostly studied biomolecules is adenosine-5′-triphosphate (ATP). Based on its vital role for energy production and storage in living cells, it can be used as an indicator for cell viability or cell damage.[1] Thus, the detection of ATP levels can be extremely useful for metabolic studies in cell biology as well as clinical diagnostics. In this sense, fluorescent nanoparticles are regarded as one of the most promising approaches for analytical applications in biology and medicine due to several of their unique characteristics, namely their size, chemical stability and versatility as well as relatively low cytotoxicity when using a biocompatible matrix.[2]

One of the most promising approaches for ATP fluorescence indicator dyes was introduced by Hamachi *et al.*,[3] who used Zn(II) complexes for the fluorescent detection of phosphorylated peptides and other species, in an attempt to mimic the binding mechanism of phosphatases to their substrates. With this in mind, our group developed a fluorescent indicator for ATP, **4·Zn**, based on naphthalimide as the fluorophore and a Zn(II) metal cation complexed with a dipicolylamine (DPA) moiety as the receptor.[4] Apart from its sensitivity to ATP, this indicator provides very favorable optical properties, such as high quantum yields and a large Stokes' shift.

The immobilization of fluorescent indicator dyes onto nanoparticles is of significant importance since plain organic dyes are often toxic to cells. Moreover, many indicator dyes only show changes in fluorescence intensity making them unreliable for quantitative measurements of analyte concentration, since effects such as dye aggregation or fluctuations in the light source intensity may influence their fluorescence. The latter problem can be overcome by immobilizing a second but inert fluorescent dye (reference) in the nanoparticles. By relating fluorescence changes of the indicator dye to changes of the reference dye, ratiometric measurements can be achieved. The success of this methodology was already demonstrated for several analytes, including pH and chloride.[5]

The design of the ATP-sensitive nanoparticles started with the synthesis of the silica core nanoparticles according to a modified Stöber method, using tetraethoxyorthosilicate (TEOS) as the precursor. The reference dye consisted of a silylated-sulforhodamine (see ESI† for details) which was also added to the reaction mixture. Consequently, the silyl ether group provided covalent attachment to the silica matrix of the nanoparticles. An undoped silica shell was deposited around the nanoparticle cores. The purpose here was to prevent any effects of Förster resonance energy transfer (FRET) between the reference and the indicator dye, as well as obtaining an homogeneous reactive surface for further functionalization.[6] The outer surface of the nanoparticles was then functionalized with amine groups using 3-aminopropyltriethoxysilane (APTEOS) which finally allowed for the covalent attachment of **4·Zn** to the surface of the nanoparticles *via* *N*-hydroxy-succinimidyl ester activation. A full scheme of the synthesis is found in Fig. 1. The size and polydispersity of the nanoparticles were determined by dynamic light scattering (DLS). In order to assess the effect on the hydrodynamic diameter of the nanoparticles, the size of the nanoparticles was measured before and after functionalization with **4·Zn** and found to be 120 and 126 nm, respectively (see Fig. S1 in ESI†). This slight difference is expected if we take into account that the size of the indicator dye as well as the alteration of chemical properties on the surface of the nanoparticles may affect their solvation sphere. Furthermore, in both cases, the polydispersity index revealed a

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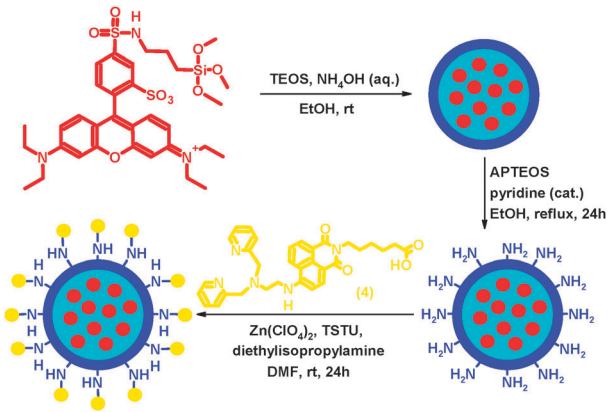

Fig. 1 Design and architecture of ATP-sensitive nanoparticles. Description: Light blue—silica core nanoparticle; dark blue—undoped silica shell; ●—reference dye; ●—indicator dye.

homogenous size distribution, with values of 0.05 and 0.07, for prior and post functionalization, respectively.

The analytical performance of the nanoparticles towards ATP was studied in aqueous buffer at pH 7.4 for different analyte concentrations using fluorescence spectroscopy. The resulting fluorescence emission spectra are shown in Fig. 2. Upon addition of 330 μM ATP to a suspension of the fluorescent nanoparticles (1.0 ± 0.2 mg mL^{-1}), an increase in the emission of the indicator dye (up to 0.5 fold) was observed while the signal of the reference dye remained almost unaffected (around 11% when compared to the fluorescence of the indicator dye). This observation is in accordance with results for the free indicator dye in solution. However, the signal changes observed for **4·Zn** when immobilized onto the nanoparticles are smaller compared to the values obtained in solution (around 2.3 fold). In fact, the apparent association constant (K_{ass}) of the fluorescent nanoparticles with ATP, which was determined by plotting the fluorescence intensity changes against the logarithm of analyte concentration (see the inset in Fig. 2), was found to be 3.3 × 10^3 M^{-1}, roughly 50 times smaller than for the free dye in solution (K_{ass}(**4·Zn**) = 1.5 × 10^5 M^{-1}).[4] This loss of sensitivity can be explained by the interaction mechanism between the indicator dye and ATP. It is assumed that this interaction is not only due to the binding of the phosphate groups in ATP to the Zn(II)–DPA receptor unit, but also due to π–π stacking effects between the adenine moiety of ATP and the naphthalimide fluorophore in the indicator dye. The immobilization of **4·Zn** onto the surface of the silica nanoparticles may introduce some sterical hindrance which prevents a more efficient binding to the analyte, especially in the case of the stacking interactions. This translates into a loss of sensitivity of the indicator dye towards ATP. Such π–π stacking effects have previously been made responsible for the differences in interaction between closely related nucleotides (which differed only in the nucleic base) and acridine bearing a Zn(II)–cyclen unit.[7] It is assumed to be the main reason for the observed selectivity towards ATP (and also ADP, as reported previously)[4] when compared with the other nucleotides having different bases (see Fig. S2 in ESI†).

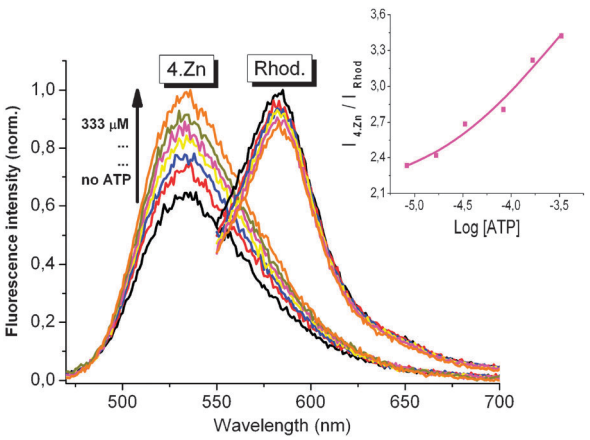

Fig. 2 Changes in fluorescence of the nanoparticles at different ATP concentrations: 0, 8, 17, 33, 83, 167 and 333 μM (λ_{exc} (**4·Zn**) = 450 nm, λ_{exc} (Rhod.) = 530 nm).

The fluorescence of the nanoparticles was also measured in the presence of other nucleotides, namely guanosine triphosphate (GTP), uridine triphosphate (UTP) and cytidine triphosphate (CTP), at three different analyte concentrations. A summary of these results is represented in Fig. 3. Although all of these nucleotides present very similar structures, differing only in the nucleobase, ATP induces a considerably stronger response in the fluorescence of the indicator dye than any of the other analytes under study. Nevertheless, the difference in sensitivity between ATP and the other nucleotides provided by the covalently immobilized indicator dye is decreased when compared to the free indicator dye in solution. This could indicate that the electrostatic interactions between the Zn(II)–DPA receptor group of the indicator dye and the phosphate groups of the analytes become more relevant than the aforementioned π–π stacking effects when **4·Zn** is immobilized onto nanoparticles. This is also consistent with the previous explanation for the recorded loss of sensitivity.

In order to confirm that the Zn(II)–DPA centre endured the synthetic procedure and was, in fact, the recognition element towards ATP, a simple experiment was performed. To a cuvette containing a suspension of nanoparticles (1.0 ± 0.2 mg mL^{-1}) with ATP (333 μM), an excess amount (1667 μM) of a complexing agent, ethylenediaminetetraacetic acid (EDTA), was added. The resulting spectra show a remarkable decrease in the fluorescence of the indicator dye, with no visible changes

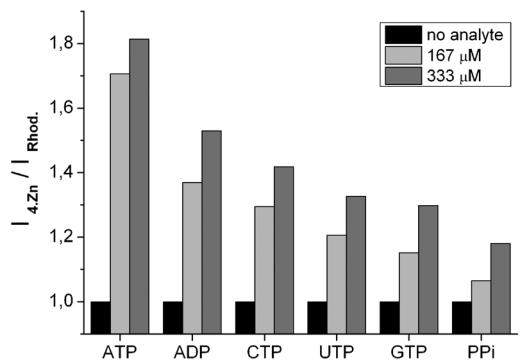

Fig. 3 Influence of analytes on the fluorescence properties of the ATP-sensitive nanoparticles. Adenosine-5′-diphosphate (ADP) and pyrophosphate (PPi) were also added to the graph for comparison.

in the reference dye (see Fig. S3 in ESI†). This result supports that Zn(II) still has been present in the indicator dye molecules which are attached to the nanoparticles' surface and that, upon removal of Zn(II) from the DPA centre using EDTA, sensitivity towards ATP is no longer observed. With these results in mind, the possibility of removal and subsequent rebinding of Zn(II) to the nanoparticles and how this process affected their sensitivity towards ATP was investigated. Proven successful, this approach would provide a mean of "recycling" the nanoparticles, suggesting their possible application in microfluidics,[8] if the nanoparticles were physically attached to such a system. Accordingly, an aliquot of nanoparticles was suspended in a vial containing an aqueous solution of EDTA and stirred overnight. The nanoparticles were then washed thoroughly with water. Upon addition of ATP to a suspension of nanoparticles, no changes were observed in the recorded spectra. However, when Zn(II) was added to the same cuvette, a considerable increase of the indicator fluorescence (0.5 fold) occurred, while the reference dye's emission remained unaltered (Fig. S4 in ESI†). This proves not only that Zn(II) was removed and rebound, but also that a full regeneration of the detection capacity of the nanoparticles towards ATP was achieved.

In order to evaluate the potential of the fluorescent nanoparticles for biomedical applications we studied cellular uptake and cytotoxic effects using epithelial normal rat kidney (NRK) cells. Nanoparticle cytotoxicity was investigated by impedimetric monitoring of the cell response during nanoparticle exposure for 48 hours. Impedimetric monitoring was the method of choice as it works label-free and the readout does not interfere with the nanoparticles' luminescence. The bar chart in Fig. S5 (ESI†) shows cell viability for different nanoparticle concentrations. Untreated cells were set to 100%, as positive control cells were treated with 0.1% (w/v) Saponin which is known to permeablize the plasma membrane leading to immediate cell lysis. Results indicate that cell viability was not impaired at all by exposure to ATP-sensing nanoparticles at concentrations ranging from 0.1 to 0.4 mg mL^{-1}.

To study nanoparticle incorporation NRK cells were incubated with 0.2 mg mL^{-1} of nanoparticles for 48 hours. Afterwards, cells were extensively washed using PBS buffer and the uptake of particles was studied by confocal laser scanning microscopy. Cells were imaged in physiological buffer at pH 7.4. Micrographs in Fig. 4 show typical optical *xy*-sections through the NRK cell bodies. The fluorescence of the rhodamine dye (a) and the naphthalimide dye (b) were recorded separately using individual excitation and emission filters. As depicted in the merged image (c) the reference and indicator dyes perfectly co-localize in the cytoplasm of the cells. Mixed colors indicate that particles are present in different cellular compartments. For a better evaluation of intracellular localization of nanoparticles a DAPI-based staining of the nucleus was performed in addition. Subsequent microscopic inspection reveals that nanoparticles are present in the cytoplasm but not in the nucleoplasm (Fig. 4d). Further research regarding nanoparticle uptake mechanism and detailed intracellular localization are currently ongoing. Since nanoparticles were incorporated in non-phagocytosing animal cells without using additional agents and without exerting cytotoxic effects, they are superior to most organic fluorophores and may pave the way to applications in biomedical studies.

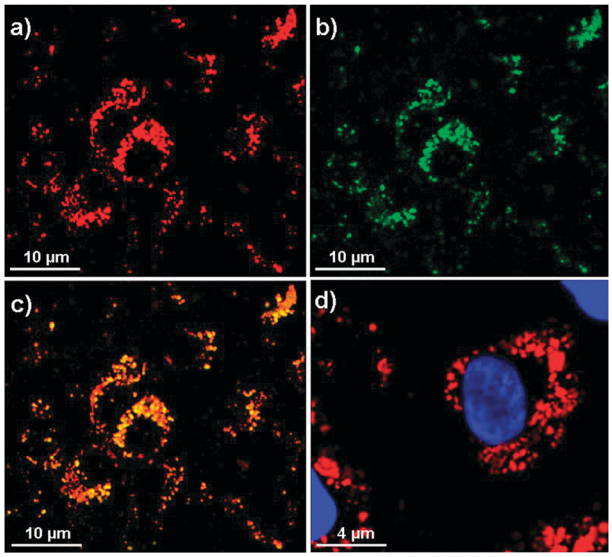

Fig. 4 Confocal fluorescence micrographs of NRK cells incubated with nanoparticles (0.2 mg mL^{-1}) for 48 hours: (a) rhodamine-reference dye, (b) naphthalimide-indicator dye, (c) merge and (d) rhodamine-reference dye after cell fixation and DAPI-nuclear staining.

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