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ORIGINAL PAPER

# Encapsulation of Hydrophobic Dyes in Polystyrene Micro- and Nanoparticles via Swelling Procedures

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**Abstract** Aiming at the derivation of a generalized procedure for the straightforward preparation of particles fluorescing in the visible and near-infrared (NIR) spectral region, different swelling procedures for the loading of the hydrophobic polarity-probe Nile Red into nano- and micrometer sized polystyrene particles were studied and compared with respect to the optical properties of the resulting particles. The effect of the amount of incorporated dye on the spectroscopic properties of the particles was investigated for differently sized beads with different surface chemistries, i.e., non-functionalized, amino-modified and PEG-grafted surfaces. Moreover, photostability and leaking studies were performed. The main criterion for the optimization of the dye loading procedures was a high and thermally and photochemically stable fluorescence output of the particles for the future application of these systems as fluorescent labels.

**Keywords** Fluorescence · Nile Red · Polystyrene · Nanoparticles · Microparticles · Encapsulation · Swelling

## Introduction

At the core of fluorescence signaling, imaging, and sensing are fluorescent labels and reporters, that are simply accessible, conveniently excitable and detectable with conventional instrumentation, bright, sufficiently stable under experimental conditions, soluble in application-relevant media, and equipped with functional groups for site-specific labeling. Advantageous are also the suitability for multiplexing and signal enhancement strategies as well as a low toxicity. Of increasing importance with this respect is the incorporation of organic or inorganic fluorophores into nano- and micrometer-sized organic and inorganic particles [1–8]. This strategy cannot only be used for different classes of fluorophores and enables straightforward signal amplification and access to multiplexing and barcoding using dye combinations [8–12], yet it elegantly circumvents the often undesired sensitivity of most chromophores to their local environment. In the case of hydrophobic dyes such as many NIR fluorophores, it presents a simple procedure for the use of these dyes also in a biologically relevant aqueous environment. In addition, fluorophore encapsulation often results in an increased fluorescence quantum yield and photostability [13–15] and can minimize undesired interactions e.g. between fluorescent reporters and abundant plasma proteins in the case of in vitro and in vivo applications. Accordingly, procedures for the preparation of differently sized fluorescent particles from various chromophores are quickly evolving as well as (bio)analytical applications of particulate labels and particle-based platforms [16–20].

Principally, nano- and micrometer-sized fluorophore-containing particles can be prepared by steric inclusion during particle formation and, synthetically more challenging, synthesis from dye-labeled monomers or precursors [3, 8, 21]. These procedures require harsh reaction conditions during the polymerization, hampering the encapsulation of sensitive fluorophores, and in the latter case also reactive dyes. This renders straightforward swelling procedures advantageous for the encapsulation of dyes, especially due to their potential for generalization, i.e., use for a broad variety of different dye classes. For this purpose, several

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polymer matrices have already been exploited, e.g., poly (methyl methacrylate) (PMMA) [22] or polystyrene [23, 24]. Particulary, polystyrene particles (PSP) present an attractive candidate, because they are commercially available with various surface chemistries in different sizes covering the nanometer- to micrometer range from 15 nm up to several micrometers. This renders PSP very versatile and attractive materials e.g. for the design of drug carriers or fluorescent reporters for imaging applications [25].

The loading of PSP with organic or anorganic fluorophores is already well established [26–28], yet comparatively little effort has been dedicated to the optimization of the swelling conditions or a systematic study of the influence of dye loading for differently sized and surface-functionalized PSP. These informations, however, are necessary for the eventually desired generalization of this approach. This encouraged us to systematically investigate and compare different swelling procedures for nano- and micrometer-sized PSP varying in surface chemistry, i.e., non-functionalized, amino-modified, and PEG (polyethylene glycol)-grafted particles, employing the well characterized polarity probe Nile Red [29–32] as model fluorophore. With this respect, we studied the influence of the amount of incorporated dye on the resulting spectroscopical properties of the PSP and optimized the swelling conditions focusing on a maximum fluorescence output.

## Experimental

### Materials

Carboxyl-functionalized polystyrene particles with sizes of 0.1 μm and 1 μm and non-functionalized plain 10 μm sized PSP, respectively, were purchased from Kisker Biotech and ultrasonically treated prior to use. 10 μm sized amino-modified and PEG-grafted PSP, purchased from Rapp Polymere GmbH, were suspended in distilled water and ultrasonically treated prior to use. The fluorescent dye Nile Red was purchased from Fluka and employed without further purification.

All the solvents (tetrahydrofuran (THF), chloroform, 2-propanol, and ethanol) were of UV-spectroscopic grade and purchased from Sigma-Aldrich Co. and used as received.

### Methods

#### *Swelling procedures*

Nile Red was dissolved in THF in different concentrations ranging from $5\times10^{-3}$ mol/L to $5\times10^{-5}$ mol/L. Dye loading of the PSP was performed by addition of 100 μL of a Nile Red-containing solution to 600 μL of an aqueous suspension of the PSP (0.5 weight percent (w%)). If not otherwise stated, the occasionally shaken suspension was centrifuged (Eppendorf centrifuge 5415D; 100 nm PSP at 15,000 g for 20 min, 1 μm PSP at 5,000 g for 10 min, 10 μm PSP at 4,000 g for 10 min) after 30 min. The accordingly separated PSP were washed twice with distilled water.

The same procedure was also carried out in chloroform substituting THF. In this case, prior to the addition of the dye solution, the PSP provided by the manufacturer as aqueous suspension, were centrifuged and washed twice with 2-propanol to remove water.

To optimize the swelling conditions with respect to the amount of incorporated dye, different stock solutions of Nile Red were prepared to assure an amount of dye n of $1\times10^{-7}$ mol per sample regardless of the volume fraction of the solvent. The dye solutions were added to the particle suspensions, each containing 3 mg of PSP. In the case of the time-dependent swelling studies, the end of the centrifugation process was assumed to be the end of the dye incorporation process, since then, the PSP are supposed to have only little contact with the dye containing supernatant.

#### *Determination of the amount of incorporated dye*

For the determination of the amount of PSP-incorporated dye, the PSP suspensions were centrifuged (15,000 rcf, 20 min) and the aqueous supernatant was removed. The PSP were dissolved in pure THF followed by subsequent measurement of the absorption spectra of the transparent THF solutions. The average amount of dye incorporated into the 3 mg PSP employed for the dye loading studies was calculated from the absorbance measured at the dye's longest wavelength absorption maximum, using the Lambert-Beer law and the previously determined molar absorption coefficient of Nile Red in THF in the presence of dissolved 0.1 w% polystyrene.

#### *Absorption and fluorescence spectroscopy*

Prior to each spectroscopic measurement, the washed PSP were transferred to fluorescence cuvettes and suspended in 3 mL distilled water, resulting in a polystyrene concentration of 0.1 w%.

Absorption measurements were carried out on a Cary 5000 UV-Vis-NIR spectrophotometer and fluorescence measurements on a Perkin Elmer LS50B fluorometer in a 90° standard geometry. For all the measurements, the temperature was kept constant at 298±1 K.

For the determination of the molar absorption coefficients of Nile Red in THF, different dilute dye solutions, originating from two stock solutions, were measured in the presence of 0.1 w% polystyrene. The dye concentration was varied from $5\times10^{-6}$ mol/L to $1\times10^{-5}$ mol/L.

The presented fluorescence emission spectra are not corrected for the spectral responsivity of the fluorometer´s emission channel, thus presenting instrument-specific data [33].

*Confocal laser scanning microscopy*

Photostability studies were performed with a confocal laser scanning microscope Olympus FluoView™ FV1000 (Olympus, Hamburg, Germany) using a UPLSAPO 40× objective (N.A. 0.9). The excitation wavelength used was the 488 nm line of the argon ion laser which was reflected by a beamsplitter BS 20/80. The emission was detected in the wavelength range from 550 nm to 650 nm. Photostability data were recorded using the Time Controller Mode function of the CLSM, which permits monitoring of time-lapse experiments. The particles were illuminated with the 488 nm line for 1 min (70% laser power, pixel time 20 μs/pixel). Confocal images were taken using the same 488 nm line with 2.5% laser power and pixel time 20 μs/pixel in 512×512 pixels. Regions of interest (ROIs) of 150 $\mu m^2$ were selected to evaluate the time dependence of the fluorescence intensity.

## Results and discussion

For the systematic investigation of swelling procedures to incorporate fluorophores into PSP, we used the photophysically well studied fluorescent reporter Nile Red, absorbing and emitting in the visible region, due to the sensitivity of its absorption and emission properties to the polarity of its microenvironment [29–31]. In apolar media, this dye displays a noticeable spectral overlap of its absorption and emission bands, making it prone to concentration-dependent reabsorption effects as typically encountered with the majority of NIR dyes. Moreover, Nile Red is hydrophobic [34] and only negligibly soluble in water. Furthermore, in the presence of increasing amounts of water, its absorption and emission bands are red-shifted and its fluorescence quantum yield decreases dramatically (Fig. 1). These effects provide an additional tool for the evaluation of potential interactions between encapsulated dye and surrounding solvent, i.e., water molecules.

Optimization of the swelling conditions

In order to optimize and generalize the swelling conditions, important parameters affecting the encapsulation procedure in PSP were studied. First of all, suitable solvent systems have been investigated, since there exists a broad variety of dyes with different solubility. To get access to several swelling media, we compared THF and chloroform, since

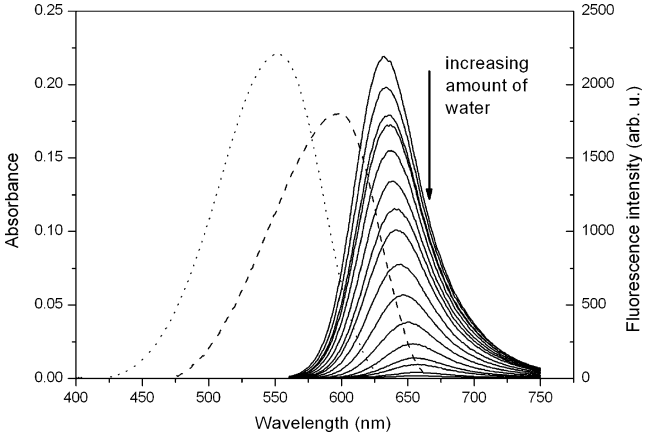


**Fig. 1** Absorption spectra of Nile Red in ethanol (dotted line) and in a 10% ethanol/water-mixture (dashed line). Absorption-weighted fluorescence emission spectra (solid lines) of Nile Red in different ethanol/water-mixtures ranging from 100% ethanol to 5% ethanol / 95% water; excitation was at 510 nm

both are common solvents for most of the available fluorophores. In Fig. 2, the parameters swelling time and solvent composition (mixtures of THF and water as well as chloroform and 2-propanol, respectively, varying in volume fractions) are shown, exploiting the amount of encapsulated Nile Red as a measure for the efficiency of the incorporation procedure. In the left panel of this figure, the influence of the THF content and the swelling time are shown. The right panel displays a comparison of the two solvent mixtures. The relative standard deviation for dye loading, i.e., for the amount of dye incorporated in the PSP, has been previously determined to about 5% from five independent measurements (data not shown). In the case of solvent mixtures containing THF, volume fractions exceeding 14% of this solvent lead to a decrease in the amount of incorporated dye. For this solvent composition as well as for higher volume percentages of THF, the swelling time does not have an influence on the dye incorporation rate and the equilibrium between Nile Red dissolved in PSP and in the solution is already reached within 15 min. This is most likely due to the very good solubility of Nile Red in THF, affecting the diffusion process. Interestingly, a higher amount of THF in the swelling solvent does not seem to result in a higher amount of encapsulated dye. For THF volume fractions below 14%, we observed the highest incorporation rates ranging from 75% to 95% and a moderate dependence on swelling time. Here, the incorporation process is practically completed within 30 min.

In contrast, for the chloroform/2-propanol solvent system (Fig. 2, right panel), we could not detect any dye incorporation, regardless of the chloroform-to-2-propanol volume ratio. This is most likely due to the very good solubility of the dye in this solvent mixture hampering a dye incorporation in the polystyrene matrix.

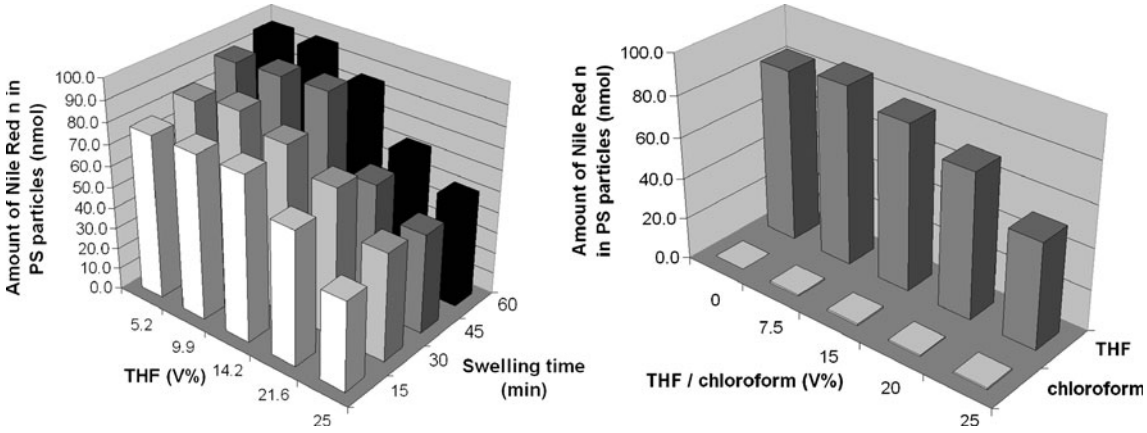


**Fig. 2** Left: Amount of incorporated dye in carboxylated PSP (1 μm) as a function of the volume fraction of THF in the swelling solution and of the swelling time. The amounts of dye n in the swelling experiments were $1\times10^{-7}$ mol in each sample. Right: Comparison of different solvent system (THF/water and chloroform/2-propanol) used for loading experiments with respect to the amount of incorporated dye at different volume fractions for a swelling time of 30 min

It should be noted here, that a volume fraction of 14% THF has been identified as a good compromise between the relative amount of PSP-incorporated dye and the amount of dye solution present. This can be especially important for less soluble dyes, where the preparation of highly concentrated stock solutions can be problematic. These findings underline the importance of the solubility of the dye in the solvent mixture used for PSP swelling as compared to its solubility in the polystyrene matrix. Obviously, the balance between the solubility in the different matrices seems to be the most important parameter controlling dye incorporation for this encapsulation procedure, leaving the swelling of the polystyrene matrix only subordinate.

## Influence of the particle size on dye loading

For the investigation of the influence of the particle size on the amount of incorporated dye, we studied the Nile Red loading of PSP, sized 0.1 μm, 1 μm, and 10 μm, respectively. These experiments based on the same swelling protocols using different dye concentrations between $5\times10^{-7}$ mol to $1\times10^{-8}$ mol with a THF content of 14 V%, which was previously found to present an optimum (Fig. 2). As shown in Fig. 3, for each particle size, we observed a good correlation between the amount of Nile Red in the swelling solution and in the PSP. The ratio of the dye, dissolved in the swelling solution and in polystyrene, depends on the particle size or particularly on the volume-to-surface ratio. The amount of incorporated dye increases constantly with dye concentration until a saturation of the polystyrene matrix occurs. Generally, in the PSP, very high Nile Red concentrations can be realized (see Table 1).

## Spectroscopic properties of Nile Red-loaded PSP

### *Amount of incorporated dye*

The rigid and apolar polymer matrix is supposed to have a considerable influence on the spectroscopic properties and photochemical stability of a fluorophore [13–15, 35, 36], especially in the case of a polarity probe like Nile Red [29–32]. The fluorescence emission spectra of PSP, loaded with various amounts of Nile Red, are summarized in the left panel of Fig. 4. Upon PS-encapsulation, blue-shifted

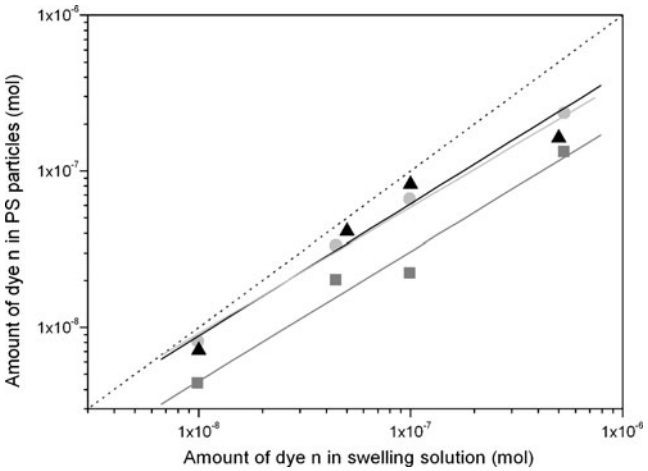


**Fig. 3** Amount of incorporated dye n as a function of the dye concentration used for swelling of PSP (3 mg) of different sizes: 100 nm (dark grey squares), 1 μm (light grey circles), 10 μm (black triangles). The lines are only a guide to the eye. The theoretical maximum amount of dye for 100% incorporation is also indicated (dashed line)

**Table 1** Rate of incorporation of Nile Red in PSP

| Particle Size (nm) | Amount of dye in swelling solution (nmol) | Amount of dye in 1 mg PS (nmol) | Dye molecules per particle | Relative incorporation |
|---|---|---|---|---|
| 100 | 9.9 | 1.5 | $4.8\times10^{2}$ | 44% |
| | 44.5 | 6.7 | $2.2\times10^{3}$ | 45% |
| | 99.0 | 7.4 | $2.5\times10^{3}$ | 23% |
| | 530.0 | 44.4 | $1.5\times10^{4}$ | 25% |
| 1,000 | 9.9 | 2.7 | $9.0\times10^{5}$ | 82% |
| | 44.5 | 11.1 | $3.7\times10^{6}$ | 75% |
| | 99.0 | 22.1 | $7.3\times10^{6}$ | 67% |
| | 530.0 | 78.6 | $2.6\times10^{7}$ | 45% |
| 10,000 | 10.0 | 2.4 | $7.8\times10^{8}$ | 71% |
| | 50.0 | 13.7 | $4.5\times10^{9}$ | 82% |
| | 100.0 | 27.3 | $9.0\times10^{9}$ | 82% |
| | 500.0 | 54.1 | $1.8\times10^{10}$ | 32% |

absorption and emission spectra were observed which resemble the spectra of Nile Red in an aprotic and apolar solvent like dibutylether (BOB). This matrix-induced effect is indicative of the relatively low polarity of polystyrene and the almost entire absence of water molecules in the local environment of the dye pointing to complete dye encapsulation (see Fig. 1). This is also suggested by the spectral position and shape of the absorption and emission bands in PSP as compared to the spectra found in ethanol/water mixtures. The observed red shift of the Nile Red emission maximum, that amounts to about 20 nm when comparing the emission spectra of the PSP loaded with the lowest and the highest amount of dye (Fig. 4, left panel), is ascribed to reabsorption. Simultaneously, the fluorescence intensity decreases for a Nile Red loading exceeding $4.5\times10^{-8}$ mol dye per 3 mg PSP (Fig. 4, right panel). This points to the occurrence of fluorescence quenching dye-dye interactions within the PSP although the absorption spectra did not provide a hint for aggregate formation. Furthermore, reabsorption effects cannot be excluded. Admittedly, particle-related scattering may hamper the monitoring of dye-concentration-dependent changes in the absorption band. Regarding this complex interplay, for the desired realization of bright PSP, the optimum concentration needs to be identified for each dye as the occurrence of reabsorption and fluorescence selfquenching are at least partly dye-inherent properties [37].

It should be noted, that all the dye-loaded PSP generated and presented in this study, display high fluorescence intensities when suspended in water.

*Influence of particle surface chemistry*

Surface functionalization of the fluorescent PSP is the prerequisite for the use of these particles as fluorescent labels, imaging tools, and targeted reporters or sensors [37]. Similarly important for biological applications and especially for in vivo molecular imaging is the considerable impact of the surface chemistry on particle toxicity [38].

To assess the influence of PSP surface chemistry on the incorporation and spectroscopic properties of Nile Red, we loaded plain and amino-modified 10 μm-sized particles with the same amount of Nile Red. In addition, we performed similar experiments with amino-modified PSP grafted with PEG spacers that are supposed to improve the reactivity of

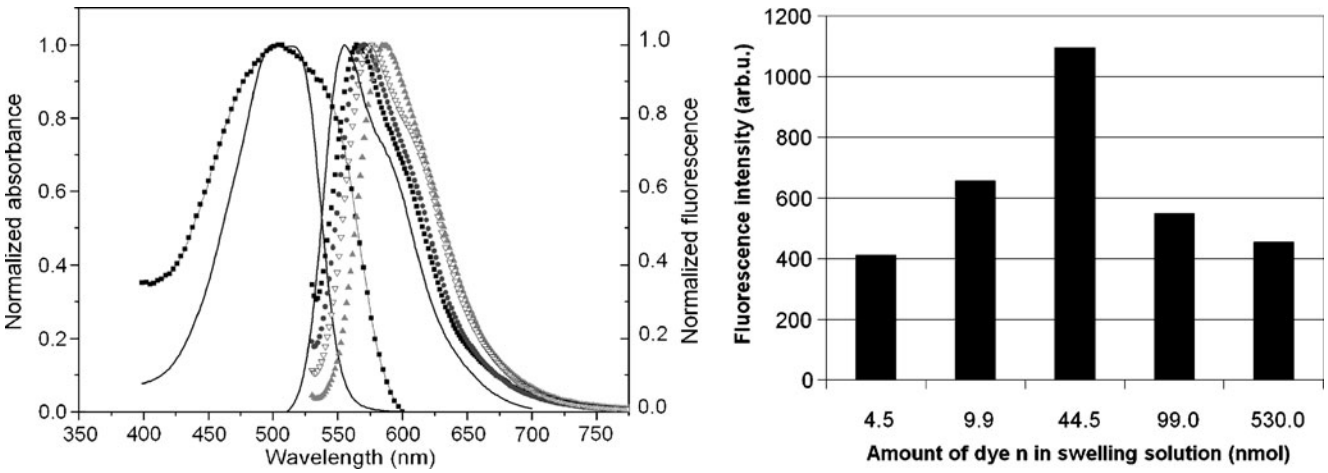


**Fig. 4** Left: Normalized absorption and fluorescence (solid lines) spectra (excitation at 510 nm) of Nile Red in dibutylether as well as the normalized absorption (linked squares) and emission (symbols) spectra of encapsulated Nile Red. PSP were stained with different amounts of dye: $5\times10^{-9}$ mol (squares), $1\times10^{-8}$ mol (circles), $1\times10^{-7}$ mol (full triangles), and $5\times10^{-7}$ mol (open triangles). Right: Relative fluorescence intensities of the differently loaded PSP

these functionalities because the reactive sites, which are located at the end of the spacer arms, behave kinetically like in solution (www.Rapp-polymere.com). The obtained emission spectra of the solvatochromic Nile Red are summarized in Fig. 5, highlighting the obvious influence of particle functionalization on the spectral position of the emission maximum. These measurements reveal significant bathochromic shifts between the plain and the functionalized particles of 40 nm (amino-modified particles), and 55 nm (PEG-grafted amino-modified particles), respectively. The extent of these spectral changes clearly exceeds the reabsorption-related shifts shown in the left panel of Fig. 4. Concomitantly with the red shift in emission, we observed a decrease in fluorescence intensity (not shown). These findings agree well with the reported behavior of Nile Red in polar environments [29–31] and the spectral and intensity effects observed by us for this dye in ethanol/water mixtures (see Fig. 1).

## Photostability studies

The decrease of the Nile Red fluorescence intensity observed for highly loaded PSP is supposed to be related to fluorescence selfquenching within these particles (Fig. 4, right panel). To verify this assumption, we performed photostability studies with two polystyrene particles of identical size, yet different dye loading using the 488 nm laser line of a CLSM. The observed time course of the fluorescence intensities for highly loaded and slightly loaded PSP are shown in Fig. 6.

In the case of slightly loaded PSP, the fluorescence intensity shows the expected behavior of a decrease with increasing illumination time due to photobleaching of Nile Red. Highly loaded PSP display an initial increase in emission intensity, reaching a maximum value after about 5 min, followed by a subsequent continuous fading of fluorescence. This special type of "photobrightening" is ascribed to photodecomposition of dye molecules, thereby reducing dye loading and thus reabsorption and fluorescence selfquenching effects.

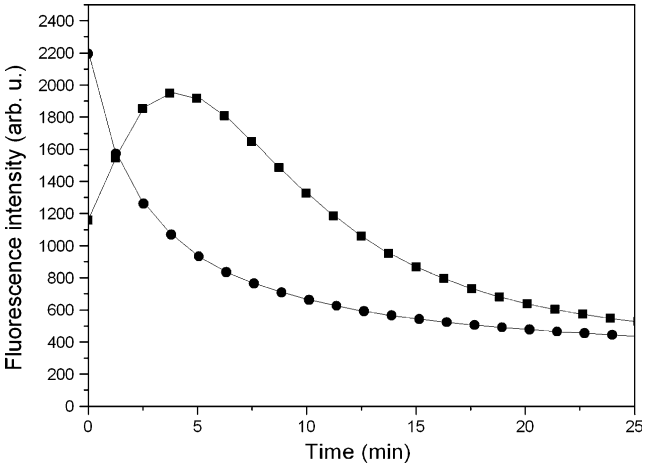


**Fig. 6** Temporal evolution of the fluorescence intensity of PSP (10 μm) loaded with different amounts of Nile Red upon illumination with the 488 nm laser line of a CLSM setup: Highly loaded PSP (squares) and slightly loaded PSP (circles)

## Leaking studies

One of the main concerns of PSP staining via a swelling procedure in comparison to the covalent attachment of fluorophores is the potential risk of dye leaking, which reduces the longterm stability of particle-based sensors or

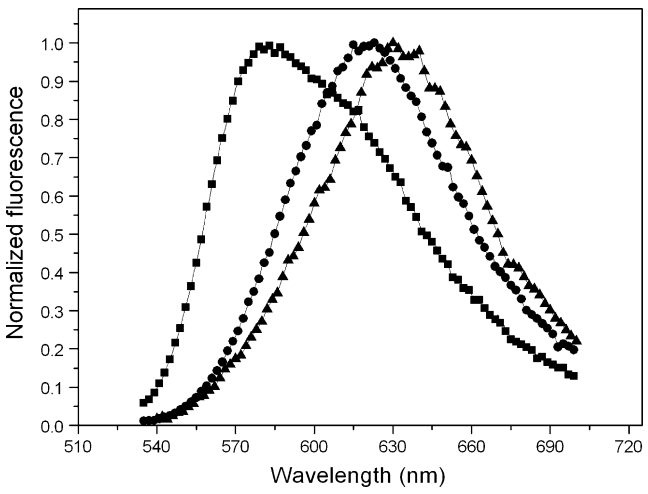


**Fig. 5** Normalized fluorescence emission spectra (excitation at 510 nm) of Nile Red in PSP (10 μm) varying in surface chemistry: non-functionalized (squares), amino-modified (circles), and PEG-grafted with amino groups (triangles)

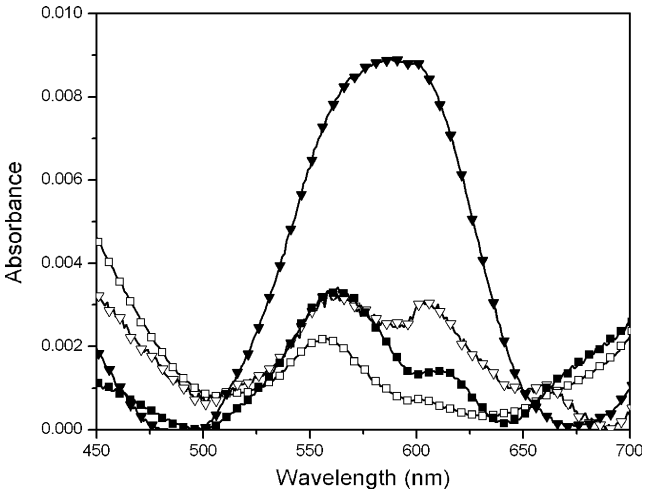


**Fig. 7** Leaking experiments of Nile Red-stained 1 μm-sized PSP demonstrated by absorption spectroscopy. The absorption spectra of the supernatant of PSP kept in pure water (hollow symbols) and in a 10% ethanol / 90% water-mixture (solid symbols) were measured at 30 min (squares) and after 3 h (triangles)

reporters. For in vivo applications, dye leaking is also critical with respect to dye cytotoxicity that needs to be determined in addition to particle cytotoxicity, if leaking under application-specific conditions cannot be excluded. The main factors governing undesired dye leaking are the hydrophilicity of the dye and its solubility in the solvent or matrix surrounding the PSP.

Aiming an intended application of the studied fluorescent PSP as targeted in vitro and in vivo probes, we performed leaking tests with Nile Red-loaded PSP, stored in pure distilled water and in a 10% ethanol/water mixture. After particle separation by centrifugation at different storage times, the absorption spectra of the supernatant were measured (see Fig. 7). These experiments simulate comparatively harsh conditions, since the solubility of Nile Red in ethanol considerably exceeds e.g. its solubility in commonly used BSA/PBS solutions, typically studied as model systems for body fluid [39]. While we observed only minor dye absorption in the aqueous supernatant, possibly caused by particle residues, in the 10% ethanol/water mixture, significant dye absorption was detected after 3 h. This is equivalent to about $4.5\times10^{-10}$ mol and implies a loss of ca. 1.5% of the initial amount of dye in PSP.

## Conclusions

As demonstrated for the lipophilic and solvatochromic fluorophore Nile Red, the staining of PSP with a hydrophobic dye via a THF/water swelling procedure is an elegant, versatile, and generalizable method for chromophore encapsulation, rendering such fluorophores suitable for use in biologically relevant aqueous media. With this approach, particles with sizes in the nano- to micrometer range and various surface chemistries can be easily loaded with different amounts of dye, yielding bright, and robust fluorescent reporters for a broad variety of applications. Most likely, other hydrophobic dyes can easily be encapsulated by these swelling procedures, making this an attractive method for the emerging field of particle based applications and opening the field for otherwise less attractive hydrophobic dyes emitting in the bioanalytically favored NIR wavelength region. For maximum signal generation, the swelling conditions have to be optimized. Particularly, the composition of the solvent mixture and the dye concentration have to be adjusted to specific dye properties like solubility and aggregation tendency.

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