Facile doping of silica nanoparticles with far-red cyanine dyes using the microemulsion method.

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ABSTRACT

Highly fluorescent cyanine dyes are used extensively in bioanalytical chemistry. We prepared silica nanoparticles (NPs) doped with two far red absorption cyanine dyes, FR670 and Cy5, using the microemulsion method. Facile loading of dyes was achieved using charged surfactants, where the electrostatic repulsion between negatively charged micelles and dyes forced the dyes into the silica NP during the self-assembly process. The effects of changing surfactant, and dye loading on NP morphology, absorption and fluorescence were also investigated. NPs doped with FR670 and Cy5 were 178 and 83 brighter than free dye labels, respectively. An immunocytochemistry experiment was performed, where a cancer cell line, SKOV3, expressing EpCAM, was stained with antibody labelled NPs. The results were compared against the same cell line stained with a commercial free dye label.

Keywords: fluorescence, silica, nanoparticles, immunocytochemistry, cancer.

1 INTRODUCTION

Commercial cyanine fluorescent dyes, such as Cy5, with high extinction coefficient / quantum efficiency and good photostability are used extensively in biomedical diagnostics [1]. However, in point of care diagnostics, where cost is a limiting factor and device specifications are lowered, clinically relevant levels of sensitivity are often not achievable using standard fluorophores. Furthermore, cyanine dyes are susceptible to high levels of photobleaching and molecular quenching that can limit applications and reproducibility. There are now several alternative fluorescent labels available, commonly referred to as second generation labels, for example, quantum dots, dye doped polystyrene / silica particles and fluorescent noble metal clusters that overcome many of these limitations [2]. In this work we use dye doped silica NPs in which the fluorophores are covalently linked to the silica matrix. These fluorophores are protected from the external environment, significantly reducing the effects of chemical and biological quenching. Furthermore, silica nanoparticles are nontoxic and are easily functionalised for bioconjugation.

There are two commonly used methods for the preparation of silica NPs, the Stöber method and the microemulsion methods. The Stöber method uses ammonium hydroxide catalysis to hydrolyse and condense a silica precursor in a water / ethanol mixture. In the microemulsion method, the silica hydrolysis and condensation reaction occurs at the interface of a micelle stabilized water droplet inside an oil phase. The microemulsion system provides additional control over the synthesis, dye doping and surface functionalization of silica NPs, for example, the size of the particle can be controlled through variation of water to surfactant ratio, ammonia concentration, structure of surfactant, and alkane chain length of organic solvent. The general mechanism of formation of silica NPs using the microemulsion method can be divided into 5 steps: 1, silicate precursor phase transfer; 2, hydrolysis; 3, condensation; 4, nucleation; and 5, growth (Figure 1).

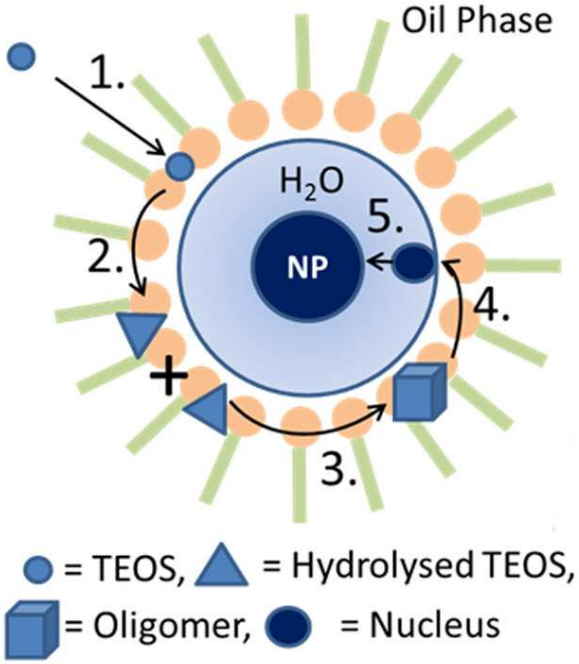

Figure 1: Mechanism of silica NP formation using the microemulsion method.

Previously Cy5 had not been encapsulated into silica NPs using conventional techniques [3]. It was postulated that Cy5 is not stable inside a negatively charged silica

matrix because hydrophilic Cy5 is negatively charged, due to the presence of sulfonic acid groups. In previous work, to incorporate Cy5 into silica NPs without dye leaching, the dye had to be conjugated to a positively charged protein, for example, polylysine [3]. We show that it is possible to incorporated cyanine dyes into silica NPs using the microemulsion method without the need for dye conjugation to a charged polymer. A combinatorial approach was used in which three surfactants were tested: 1, polyoxyethylene (10) isooctylphenyl ether (Triton® X-100); 2, polyoxyethylene (5) nonylphenyl ether (NP-5); and 3, Dioctyl sulfosuccinate sodium salt (AOT). Triton® X-100 and NP-5 are neutral linear molecules, whereas AOT is a branched molecule containing a negatively charge sulfonate group. We observed the facile doping of silica NPs with cyanine dyes using AOT on its own or in combination with NP-5. We postulate that the negative charge on AOT repels Cy5 forcing it into the silica matrix during the self-assembly process. Using a combination of AOT and NP-5 we obtained highly monodispersed silica NPs with 100 % dye incorporation. The change in fluorescence of FR670 with changing concentration of dye added, matched closely with a model calculated using standard analytical expressions of homo Förster resonance energy transfer (HFRET) [4].

The Cy5 dye doped silica NPs were tested for detection of an ovarian cancer cell line, SKOV3. These cells contain epithelial cell adhesion molecules (EpCAM), which are commonly used as targets in immunotherapy treatment of human carcinomas. In this work we conjugated anti EpCAM to Cy5 doped NPs and performed a standard cell staining experiment. The main motivation for this work is the use of NPs to detect tumour cells.

The key features of this work are (I) optimisation of the microemulsion method for the facile doping of silica NPs with cyanine dyes, (II) a systematic characterisation and comparison of morphology, absorption and fluorescence, and (III) evaluation of performance of NPs in a cell staining experiment

2 MATERIALS AND SYNTHESIS

Water soluble, Cy5 mono-reactive NHS ester and FR670 mono-reactive NHS ester were purchased from GE Healthcare and FEW Chemicals GmbH, respectively. Purified anti-human EpCAM and Alexa Fluor®647 anti-human EpCAM (both Mouse IgG2b) were purchased from BioLegend. SCOV3 cells were obtained from American Type Culture Collection and cryopreserved in liquid nitrogen. For a full list of chemicals used please contact corresponding author.

NPs were prepared using either the quaternary or ternary microemulsion method. Table 1 summarises the different approaches used.

Exp	System	Surfactant	Solvent
A	Quaternary	Triton® X-100	Cyclohexane and n-hexanol
B	Ternary	NP5	Cyclohexane
C	Ternary	NP5 / AOT	Cyclohexane
D	Ternary	NP5 / AOT	n-hexane
E	Ternary	AOT	Cyclohexane
F	Ternary	AOT	n-hexane

Table 1: Six different microemulsion methods were tested for the synthesis of cyanine dye doped silica NPs.

The corresponding transmission electron micrograph images for experiments A to F are shown in Figure 2. Experiment C, using NP-5 in combination with AOT produced highly monodispersed silica NPs. This approach was used for the synthesis of NPs for use in cell staining experiments and optimisation of dye loading.

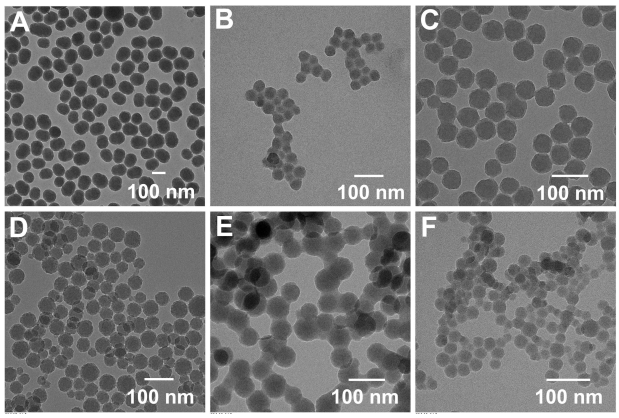

Figure 2: Transmission electron micrograph images of FR670 silica NPs prepared using the different microemulsion methods described in table 1.

The experimental protocol used for synthesis of sample C is described as follows. Anhydrous cyclohexane (0.75 mL), NP5 (0.25 mL), FR670 (1.32 mg for 2 (w/w) in silica NP) and APTMS (1.53 μL) were mixed together in a dried glass vial under a nitrogen atmosphere and stirred for 2 hours to achieve FR670 / APTMS conjugation. Separately, NP-5 (147 μL), AOT (260 mg), cyclohexane (9.75 mL) and deionised water (90 μL) were mixed together in a 15 mL plastic bottle. FR670 / APTMS conjugate solution (0.333 mL) was then added with mixing. Immediately following this, TEOS (80 μL), and 20 minutes later, ammonium hydroxide solution (60 μL, 28 (w/w)) were added and the solution stirred for 24 hours. Subsequently, TEOS (40 μL), and 30 minutes later THPMP (32 μL, 42 % in H2O), and five minutes later APTMS (8 μL) were added to activate the surface for bioconjugation. The solution was stirred for a further 24 hours. The microemulsion was then broken with the addition of ethanol (30 mL). The nanoparticles were purified by centrifugation in ethanol (3x, 15000 rpm,

8 minutes). Eight experiments were performed in total with loadings of 2.5, 2, 1.5, 1, 0.5, 0.4, 0.25, and 1 (w/w) FR670, respectively. For Cy5 dye loadings of 3.7, 2.5, 1.85, 1.5, 1, and 0.5 (w/w) were used.

Conjugation of anti-human CD326 to Cy5 NPs was performed using PAMAM dendrimers (-COOH surface groups) as multivalent linkers using a previously published protocol [5]. SKOV3 cell staining experiments were performed according to standard protocols.

3 INSTRUMENTAION

TEM micrographs were obtained using a Jeol 2100 Transmission Electron Microscope operated at 200 kV. Fluorescence measurements were performed on a Safire (Tecan) microplate reader. For Cy5 and FR670 dye doped NPs, the excitation wavelengths were set at 655 nm and 670 nm, respectively, and the emission wavelengths set at 684 nm and 700 nm, respectively. UV-Vis extinction spectra of the as-synthesized NP colloids, were measured with a Cary 50 scan UV-Visible spectrophotometer (Varian Ltd) in transmission mode. Cell images were recorded using an Olympus IX81 with an Andor iXon EMCCD camera, model 897, fluorescent microscope.

4 RESULTS AND DISCUSSION

The absorption spectra of all NPs prepared using the six microemulsion methods are shown in figure 3. All measurements were made at NP concentration of 1 mg / mL in ethanol with a path length of 2 mm. The absorption maxima at 670 nm for each sample corresponds to the amounts of FR670 dye loaded into each NP. For NPs prepared using Triton® X-100 almost no dye was loaded. Using NP-5 the amount of dye increased but was still significantly less than that loaded into samples prepared using AOT surfactant. The maximum dye absorption at 0.251 was found for NPs prepared using the ternary method with AOT and hexane oil phase. AOT differs from NP-5 and Triton® X-100 in two ways: firstly, it is a branched surfactant, and secondly it is charged. Having a branched structure equates to a significantly larger packing parameter, which for an inverse micelle corresponds to a more spherical type micelle structure. However, for the quaternary system with Triton® X-100 the cosurfactant, n-hexanol, exists in the palisade layer of the micelle, leading also to the formation of more spherical type micelles. We postulate that the negatively charged AOT in the interfacial region of the micelle generates a repulsive potential that forces the Cy5 into the growing silica particle in the core of the microemulsion. In previous work Bagwe et al investigated the loading of positively charged $Ru(bpy)_3Cl_2$ dye into silica NPs using both Triton® X-100 and AOT [6]. For NPs prepared using AOT the absorption spectra in water matched more closely with that of $Ru(bpy)_3Cl_2$ free dye in water, whereas the absorption of NPs prepared using Triton® X-100 were significantly blue shifted. The absorption spectra of Ru $Ru(bpy)_3Cl_2$ is well known to shift to the red on changing to a more polar solvent. Using similar logic to ourselves, Bagwe *et al* postulated that the positively charged $Ru(bpy)_3\ Cl_2$ located preferentially at the interface region of the micelle when using AOT surfactant. This would lead to the synthesis of a NP with dye located closer to the surface of NPs rather than in the bulk where the dye is more susceptible to changes in polarity as was observed.

In agreement with the above postulate no bathochromic shifts were observed for FR670 doped inside NPs compared to free dye in ethanol. In accordance with standard Rayleigh scattering of light by small particles the extinction spectra significantly increased as the wavelength decreased for all samples.

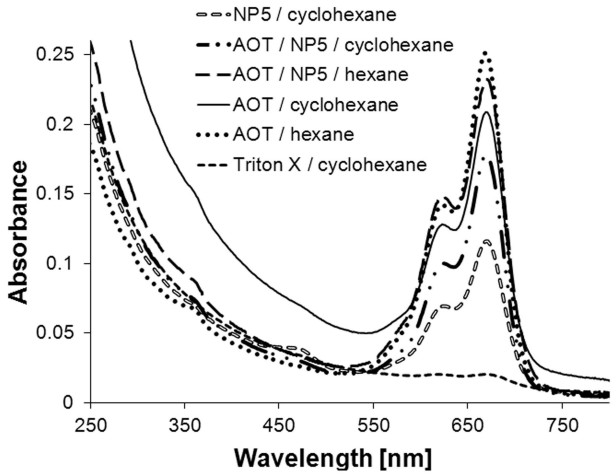

Figure 3: Absorption spectra for NPs doped with FR670 using different microemulsion methods.

A range of NPs were prepared using both AOT and NP5 surfactants with different loadings of FR670 and Cy5 from 0.25 to 3.7 (w/w) in silica. The relative fluorescence, F_R, of cyanine dye doped NPs versus free dye was determined according to the following equation,

$$F_R = \left(\frac{F_{NP}}{F_{dye}} \right)_C \qquad (1)$$

where F_{NP} is the NP fluorescence and F_{dye} is the free dye fluorescence at the same concentration, C. The maximum fluorescence for FR670 and Cy5 were 178 and 83 at loadings of 0.5 (w/w) and 1.5 (w/w) respectively. For both dyes the maximum fluorescence corresponded with a homo Forster Resonance Energy Transfer model [4]. In this model the maximum loading of dye does not continually increase with an increase in the dye loading because of self quenching losses (Figure 4).

A standard referencing method was used to determine the relative quantum efficiency, Φ, of the dye inside the NPs compared to the known Φ of the free dye. For both sets of dyes the Φ varied from 0.9 to 19 % as the

concentration of dye loaded in the particles was lowered. These values are lower than the Φ of FR670 and Cy5 at 23% and 28% respectively indicating that self quenching loses are still prevalent, even at low loadings.

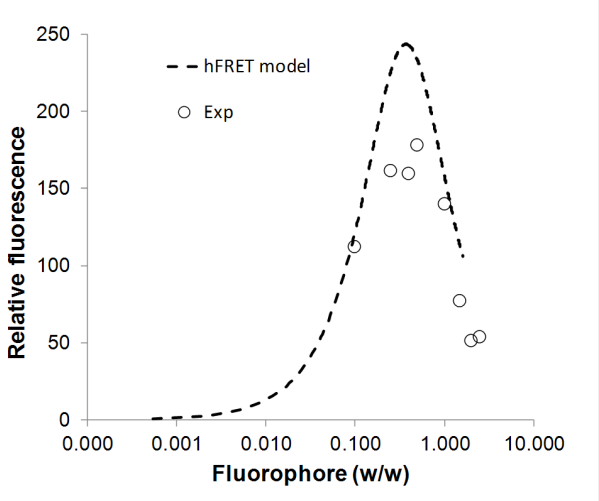

Figure 4) The change in relative fluorescence with increasing amount of FR670 (w/w) from experiment. The results are compared against a hFRET model for a nanoparticle with a radius of 50 nm.

Cell staining experiments were performed for the detection of SKOV3 cells using Cy5 dye doped silica NPs. The SKOV3 tumour cells are known to express EpCAM and therefore the NPs were labelled with a standard anti-EpCAM antibody. The fluorescence of the cells labelled with NPs were compared against a range of controls using the same instrument settings and conditions: 1, alexa fluor labelled anti EpCAM IgG; 2, anti-hIgG labelled NPs and label free NPs. In all cases the anti EpCAM labelled NPs produced significantly higher fluorescence signals (see Figure 5).

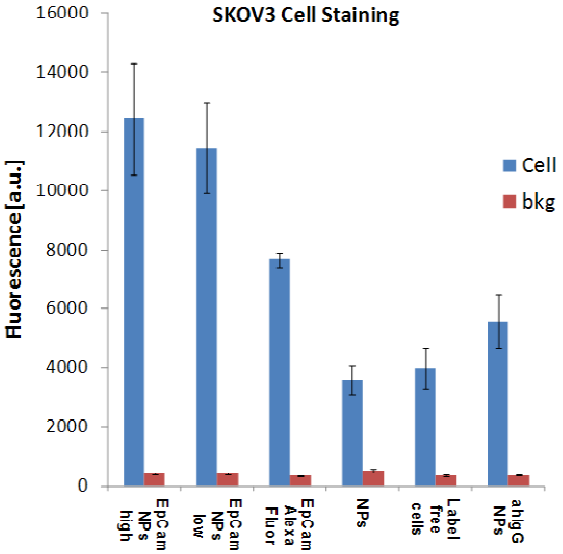

Figure 5) The fluorescence of SKOV3 cells stained with anti-EpCAM NPs and a range of controls.

5 CONCLUSION

In this work cyanine dyes, FR670 and Cy5 were successfully incorporated into silica NPs using the microemulsion method. Highly fluorescent monodispersed NPs were achieved using a combination of AOT and NP-5 surfactants. We postulate that the negative charge on AOT in the micelle generate a repulsive potential forcing negatively charged Cy5 into the growing NPs. All NPs were significantly brighter than Cy5 free dye with relative fluorescences ranging from 38 to 178. In a standard cell staining experiment of SKOV3 tumour cells NP labelled anti-EpCAM produced significantly higher fluorescent signals than a range of controls. The facile synthesis of far red dye doped silica NPs is important for the development of improved labels for the detection of pathogens in low cost point of care diagnostic devices.

6 REFERENCES

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7 ACKNOWLEDGEMENTS

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