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Fluorescent silica nanospheres for digital counting bioassay of the breast cancer marker HER2/nue

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Abstract

This paper describes the use of fluorescent silica nanospheres as luminescent signal amplifiers in biological assays based on digital counting of individual particles instead of measuring averaged fluorescence intensity. We recently described a simple method to prepare highly fluorescent mono-dispersed silica nanospheres that avoids microemulsion formulations and the use of surfactants. Modification of the Stöber method was used successfully to prepare fluorescent silica spheres with the inorganic dye dichlorotris(1,10-phenanathroline)ruthenium (II) hydrate encapsulated during the condensation of tetraethylorthosilicate in ethanol and dye aqueous mixtures. Modifications in the ammonia and water content in the reaction mixture resulted in mono-dispersed silica spheres of 65, 440 and 800 nm in diameter. The dye-encapsulating particles emit intense red luminescence when excited at 460 nm. We observed an increased photostability and longer fluorescence lifetime in our particles that we attributed to increased protection of the encapsulated dye molecules from molecular oxygen. The newly prepared fluorescent silica particles were easily modified using trialkoxysilane reagents for covalent conjugation of anti-HER2/neu. We demonstrated the utility of the fluorescent nanospheres to detect the cancer marker HER2/neu in a glass slide based assay. The assay was shown to be simple but highly sensitive with a limit of detection approaching 1 ng/mL and a linear range between 1 ng/mL and 10 μg/mL of HER2/neu.

Keywords: Breast cancer marker HER2/nue; Fluorescent silica; Stöber method

1. Introduction

Mono-dispersed fluorescent silica particles are very versatile materials that have been already applied in various bioanalytical applications (Santra et al., 2001a,b; Ji et al., 2001; Xu et al., 2001; Zhao et al., 2003; Tapec et al., 2002; Qhobosheane et al., 2001; Bagwe et al., 2003). For example, when conjugated to biomolecules they were used as cellular fluorescent probes (Xu et al., 2001; Zhao et al., 2003; Tapec et al., 2002; Santra et al., 2001a,b; Bagwe et al., 2003) and in DNA assays (Zhao et al., 2003; Tapec et al., 2002; Santra et al., 2001a). Fluorescent nanoparticles offer significant advantages compared to fluorescent dyes (Bagwe et al., 2003; Schuetz and Caruso, 2002). The encapsulation of fluorescent molecules in particles often increases their photostability and emission quantum yield due to their isolation from possible quenchers like molecular oxygen and water (Santra et al., 2001a,b; Zhao et al., 2003; Tapec et al., 2002; Schuetz and Caruso, 2002; Schardl, 2000; He et al., 2003; Zhao et al., 2004). It is also possible to functionalize the surface of fluorescent particles to facilitate their conjugation to bioactive molecules like enzymes and antibodies (Santra et al., 2001a,b; Tapec et al., 2002; Schuetz and Caruso, 2002; Schardl, 2000; He et al., 2003; Zhao et al., 2004; Azioune et al., 2004).

Covalent attachment of fluorescent dyes to silica particles has been used to prepare silica particles-based luminescent probes. The synthesis of these particles involved modification of the dyes with organoalkoxysilanes (Zhao et al., 2003; Collinson, 2002). However, most dyes are not easily modified without affecting their luminescence properties. It is also possible to physically encapsulate fluorophores in silica particles. However, the preparation of silica particles doped with organic dyes is often difficult due to the high hydrophilicity of the silica matrix and the relatively high hydrophobicity of the encapsu-

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lated dye molecules. Tan et al. recently overcame this difficulty by encapsulating hydrophilic dextran conjugates of rhodamine in silica nanoparticles (Zhao et al., 2004). They attributed the increased dye loading efficiency to hydrophobic effects. Still, despite the relatively large molecular weight-dextran used in their study the fluorophore-dextran conjugate exhibited significant leakage out of the matrix support. Tan et al. used acidic dextran to stabilize the dye in the particles through electrostatic attraction between the positively charged dextran conjugates and the negatively charged silica matrix. However, under neutral pH conditions generally required in bioanalytical applications the dextran conjugates would leak out of the silica particles.

Another limitation of synthetic methods commonly used to prepare luminescent silica particles has been the use of large amounts of surfactants in microemulsions (Qhobosheane et al., 2001; Santra et al., 2001a,b; Zhao et al., 2003; Bagwe et al., 2003; Zhao et al., 2004). Microemulsion-based techniques have been successful in producing small amounts of silica nanoparticles but scaling-up these procedures proved to be difficult. On the other hand, the well-known Stöber method (Stöber et al., 1968) is remarkable in its simplicity and does not require surface modification or addition of surfactants to achieve excellent control of size, size distribution and smooth spherical morphology of silica particles. Xia et al. (Lu et al., 2002a,b; Yin et al., 2002; Lu et al., 2004) modified the Stöber method to coat iron oxide nanoparticles, gold nanoparticles, silver nanowires and polystyrene spheres with uniform silica shells achieving controllable thickness. Core-shell silica coated cobalt nanoparticles were also prepared based on this method (Kobayashi et al., 2003). We found that the Stöber method could be used successfully to prepare fluorescent silica particles that contain the inorganic dye $[Ru(phen)_3]Cl_2$. The dye molecules were encapsulated in the particles during the condensation reaction. The paper describes the preparation of the highly luminescent mono-dispersed silica nanoparticles, their derivatization with trialkoxysilane, and their application as signal amplifiers in bioassays performed on glass slides.

The cancer marker HER2/neu was chosen as a test compound to demonstrate the utility of the silica particles as digital amplifiers in bioassays. HER2/neu is a protein which is over-expressed on the membrane of breast cancer cells. The ability to measure the levels of the HER2/neu gene and proteins is of great prognostic value since only patients that are HER2/neu positive will potentially respond to herceptin (anti-HER2/neu) based therapy. To date HER2/neu is considered the best prognostic marker for breast cancer. The levels of HER2/neu are 5.5 ng/mg protein and 185 ng/mg protein in normal breast cells and breast cancer cells, respectively (Muller et al., 2003). HER2/neu was chosen to test the utility of the digital counting assay because of its clinical importance. It is also a convenient choice since biotinylated anti-HER2/neu is available commercially and the attachment of HER2/neu to amino-modified glass surfaces was found relatively easy to perform. It is worth noting that the newly developed method of detection is highly versatile and could be used to detect many other antigens using the competitive assay method.

2. Experimental

2.1. Materials and reagents

Dichlorotris(1,10-phenanathroline)ruthenium(II) hydrate, tetraethylorthosilicate (TEOS), 3-mercaptopropyltrimethoxysilane (MPTMS) and 3-aminopropyltrimethoxysilane (APTMS) were purchased from Aldrich Chemicals, Inc. Streptavidin-maleimide and biotin-maleimide were purchased from Sigma. Human HER2/neu affinity purified biotinylated polyclonal antibody was purchased from R&D Systems. HER2/neu was purchased from Invitrogen. All reagents were used as received unless mentioned otherwise. Deionized water was prepared to a specific resistivity of at least 18 MΩ cm was used to prepare all buffer solutions.

2.2. Preparation of fluorescent silica spheres

Silica particles were prepared by adding a pre-mixed ethanol solution (25 mL) containing ammonium hydroxide and $[Ru(phen)_3]Cl_2$ aqueous solution to a TEOS solution in ethanol (5 mL) under stirring. The amount of reagents is given in Table 1. The mixture was stirred for 1 h and further sonicated for 10 min. The fluorescent nanospheres were isolated by centrifugation (5000 rpm, 10 min) and washed three times with ethanol, two times with water and again with ethanol. The samples were dried under reduced pressure and then at 100 °C for 1 h. The yield for this preparation was about 80%.

2.3. Preparation of thiol and amino-modified fluorescent silica particles

Two milligrams of fluorescent silica particles were dispersed in 10 mL of ethanol under sonication. To prepare thiol-modified particles 100 μL of 3-mercaptopropyltrimethoxysilane (MPTMS) was added to the particle solution. The particles were separated by centrifugation (5000 rpm, 10 min) and washed three times with ethanol. The particles were then re-dispersed

Table 1
Reagents and quantities used in the preparation of fluorescent silica nanospheres

TEOS (mL)	NH_4OH (30%) (mL)	$[Ru(phen)_3]Cl_2$ (0.5 mg/mL) (mL)	Ethanol (mL)	Product weight (g)	Mean diameter (from TEM images) (nm)
1.3	3	3	22.7	0.30	800 ± 20
1.3	7	3	18.7	0.30	440 ± 18
2	1.5	0.5	25	0.50	65 ± 8

in 4 mL of phosphate buffer at pH 7.4 and stored at 4 °C until use.

2.4. Preparation of streptavidin-modified fluorescent silica particles

The thiol-modified fluorescent silica particles (1 mL stock solution) were mixed with 5 mL phosphate buffer solution at pH 7.4 that contained 0.25 mg of maleimide labeled streptavidin. The mixture was incubated at room temperature under gentle stirring for 2 h. The streptavidin-labeled particles were separated by centrifugation (5000 rpm, 10 min) and washed three times with phosphate buffer solution. The particles were then re-dispersed in 4 mL of phosphate buffer at pH 7.4 and immediately used for biotin-avidin assays.

2.5. Preparation of biotin-modified glass slides

Microscope glass slides were treated with 1 M HNO_3 aqueous solution, then water and then ethanol by sonication for 15 min each, to remove deposited organic material. The pre-cleaned glass slides were blow-dried with a stream of dry-nitrogen then incubated with 3-mercaptopropyltrimethoxysilane (MPTMS) in toluene solution (5% v/v) for 4 h at room temperature. The slides were washed with toluene and ethanol and then dried under a stream of nitrogen followed by thermal treatment at 100 °C for 16 h. Then, the thiol-modified glass slides were placed in flasks containing 0.5 mg of maleimide labeled biotin dissolved in 10 mL of phosphate buffer solution at pH 7.4. Following 2 h incubation at room temperature, the biotin labeled glass slides were washed with the phosphate buffer and then used for biotin-avidin assays.

2.6. Binding of streptavidin-modified fluorescent silica particles to biotin-modified glass slides

About 100 μL of streptavidin-modified fluorescent silica particles solutions (concentration range 0–200 μg/mL) were placed on the surface of biotin-modified glass slide. The slides were incubated for 30 min at room temperature and washed several times with water and then dried under a stream of nitrogen. The fluorescent particles attached to the glass slides were counted using our digital imaging microscopy system.

2.7. Preparation of HER2/neu-modified glass slides

Amino-modified glass slides were prepared by first rinsing the slides with 1 M NaOH and then de-ionized water. The slides were then incubated in a solution of 3-aminopropyltrimethoxysilane (APTMS) overnight and washed with a phosphate buffer solution at pH 7.4. The amino-modified glass slides were incubated in a 5 mL PBS at pH 7.4 that contained 40 mM EDC/5 mM NHS and 0.25 mg of HER2/neu overnight at 37 °C under gentle stirring. The slides were then washed off un-reacted HER2/neu and used immediately in HER2/neu digital assays.

2.8. Preparation of anti-HER2/neu labeled silica particles

The streptavidin-modified fluorescent silica particles (1 mL stock solution) were mixed with 5 mL phosphate buffer solution at pH 7.4 that contained 0.25 mg of biotinylated anti-HER2/neu. The mixture was incubated at room temperature under gentle stirring for 2 h. The anti-HER2/neu labeled particles were separated by centrifugation (5000 rpm, 10 min) and washed three times with PBS at pH 7.4. The particles were then re-dispersed in 4 mL PBS at pH 7.4 and immediately used for binding assays.

2.9. Characterization of the fluorescent silica particles

Transmission electron microscopy (TEM) images of the particles were taken using a JEOL-2010 electronic microscope operating at an accelerating voltage of 200 kV. Samples for TEM were prepared by placing a drop of an aqueous particles solution on a copper grid. The particle size distribution was estimated from the measurement of about 200 particles found in an arbitrary chosen area in enlarged images. Fluorescence emission measurements were performed in a quartz cuvette using a PTI Quanta Master fluorescence spectrometer equipped with a 75 W Xenon short-arc lamp as a light source. Photostability measurements were performed in a light exposure and weathering Suntest CPS+ instrument equipped with a 1.5 KW Xenon lamp.

2.10. Digital fluorescence imaging microscopy system

Fluorescence images of the fluorescent particles attached to glass slides were taken using a digital fluorescence imaging microscopy system that consisted of an inverted fluorescence microscope (Olympus IX70) equipped with a 100 W mercury lamp as a light source. The fluorescence images were collected through a 20× microscope objective using a 460 ± 10 nm band pass excitation filter, a 505 nm dichroic mirror and a 515 nm long pass emission filter. A high-performance ICCD camera (Princeton Instruments, model BH2RFLT3) was employed for digital imaging.

3. Results and discussion

3.1. Structural characterization of the fluorescent silica nanoparticle

The synthetic procedure used to prepare the highly fluorescent silica nanospheres involved the condensation of tetraethylorthosilicate in ethanol and water mixtures based on the procedure first reported by Stöber (Stöber et al., 1968). The inorganic dye $[Ru(phen)_3]Cl_2$ was incorporated into the silica particles during the condensation reaction. This one-pot simple methodology enabled the preparation of highly fluorescent mono-dispersed silica particles with high yield of approximately 80% while avoiding the use of microemulsion formulations or surfactants. Transmission electron microscopy images of the silica particles are shown in Fig. 1. Slight modifications of the

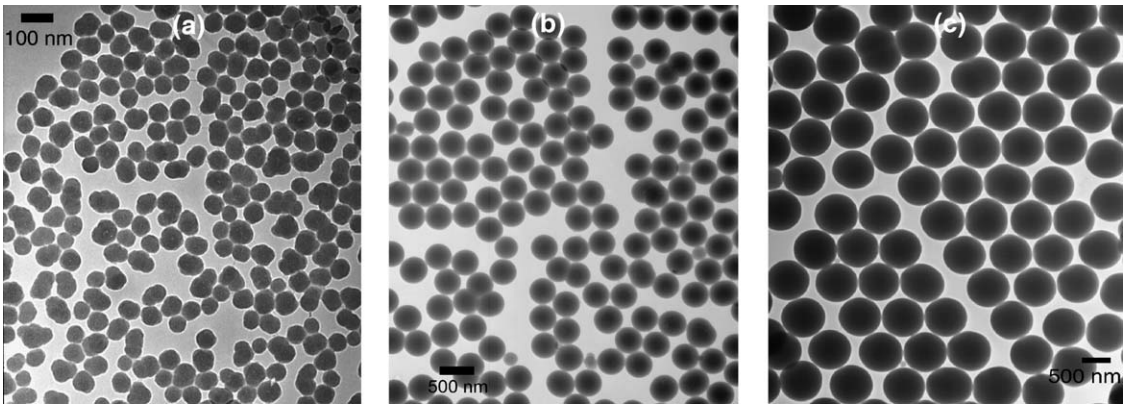

Fig. 1. Transmission electron microscopy (TEM) images of $Ru(phen)_3$ containing silica particles prepared using the Stöber method. In image (a) the scale bar is 100 nm. In images (b) and (c) the scale bar is 500 nm.

ammonia and water content in the reaction mixture (see Table 1) resulted in mono-dispersed silica spheres of 65 ± 8 nm (Fig. 1a), 440 ± 18 nm (Fig. 1b) and 800 ± 20 nm (Fig. 1c). The particles were highly mono-dispersed with smooth morphology and narrow size distribution. The surface/bulk ratio of the particles is higher in smaller particles, which increases the availability of surface binding sites in particle suspensions. However, the visualization of individual particles using our diffraction limited fluorescence microscopy instrumentation was easier due to high signal to noise ratio for particles larger than 400 nm.

3.2. Luminescence properties of the fluorescent silica nanoparticles

The $Ru(phen)_3$-encapsulating particles emitted intense red light when excited at 460 nm due to the presence of the ruthenium dye. The emission properties of the fluorescent silica particles were similar to the emission properties of free $Ru(phen)_3$ ($\lambda_{exc.} = 460$ nm, $\lambda_{em} = 590$ nm). Photostability measurements of the fluorescent silica particles were carried out by continuously illuminating particles samples with a 1.5 KW Xenon lamp. Fig. 2 shows the results of measurements comparing the photostability of fluorescent silica particles that were prepared by the Stöber method (curve a), by loading $Ru(phen)_3$ into pre-prepared silica particles (curve b), and a 0.1 mM aqueous solution of $Ru(phen)_3$ (curve c). It can be seen that particles prepared by the Stöber method were highly photostable. In contrast, the photobleaching rate of fluorescent silica particles that were prepared by dye loading was comparable with the photobleaching rate of free dye solutions. This indicated that loading dye molecules into pre-prepared particles produced fluorescent particles in which most of the dye molecules were adsorbed to the silica surface rather than encapsulated in the silica matrix. This is a reasonable explanation because of the high density of negative charges on the silica surface under neutral pH conditions and the positive charges of the ruthenium dye. On the other hand, the Stöber-like synthetic technique resulted in efficient encapsulation of the dye molecule in the silica particles. The increased photostability of the newly prepared particles was attributed to poor permeability of molecular oxygen into the silica particles. It should be noted that molecular oxygen is often required for dye photobleaching to occur since it is a precursor for singlet oxygen, which is known to trigger photodecomposition reactions. It is interesting to note that ruthenium diimine complexes like the one used in this study have been used frequently in oxygen sensing applications (Muller et al., 2003; Zhang et al., 2002). The fluorescence of these complexes increases in an oxygen free environment by an order of magnitude due to decreasing oxygen quenching efficiency (Xu et al., 2001; Zhang et al., 2002; Tang et al., 2003). However, when encapsulated in silica particles the dye did only minimally respond to changes in oxygen levels. The fluorescence intensity of these particles decreased by only 30% in oxygenated solution compared to the fluorescence of the same particle suspension in a nitrogenated solution. In contrast, the fluorescence intensity decreased by 65% for fluorescent silica particles that were prepared by dye loading into pre-prepared silica particles and by about 81% for the free dye.

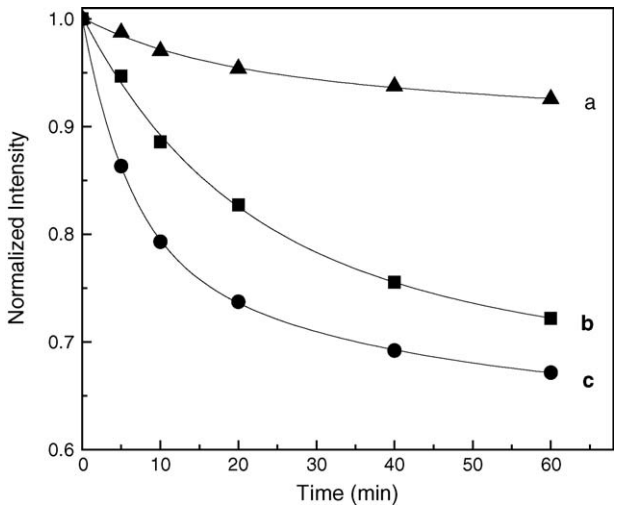

Fig. 2. Photostability measurements: (a) fluorescent silica particles prepared using the one-pot Stöber method, (b) fluorescent silica particles prepared by loading $Ru(phen)_3$ into pre-prepared silica particles, and (c) a solution of 0.1 mM $Ru(phen)_3$. The samples were excited with a 1.5 KW Xenon lamp in a Suntest CPS+ Instrument.

3.3. Surface functionalization of the fluorescent silica nanoparticles

Silica is a very versatile matrix particularly because of its inert chemical properties, optical transparency, and the relative ease of its derivatization with functional groups like amino ($–NH_2$), chloride (–Cl), carboxylic (–COOR) and thiol groups (–SH). Functionalization of silica particles was previously carried out using silane chemistry with commercially available organosilicon reagents (Bagwe et al., 2003; Azioune et al., 2004; Steinle et al., 2002; Beck et al., 1999; Shimada et al., 2003; Liu et al., 2004; Halliwell and Cass, 2001). In our study we successfully modified the surface of the fluorescent silica particles with thiol groups through hydrolysis and condensation with 3-mercaptopropyltrimethoxysilane (MPTMS) following a procedure developed by Cass et al. (Halliwell and Cass, 2001). Thiol derivatization was selected because thiol is a reactive functional group that enables covalent coupling of maleimide-modified biomolecules through the formation of thio–ether bonds. As an example, we attached maleimide-labeled streptavidin to the thiol-modified surface of the fluorescent silica particles to form streptavidin-modified fluorescent particles. The presence and activity of streptavidin on the particles surface was confirmed by attaching the particles to biotin-modified glass slides as described in Section 2. Fig. 3 shows a series of images of increasing concentration of streptavidin coated fluorescent silica particles on biotinylated glass slides. Image (a) shows the results of a control experiment in which a solution of 125 μg/mL of thiol-modified fluorescent particles was used instead of avidin modified particles. The image shows negligible non-specific absorption of fluorescent silica particles to the glass surface. Images (b–f) show the results obtained when solutions of increasing concentrations of particles were incubated with biotinylated glass slides. The concentrations of the particles were 6.25 μg/mL (image b), 12.5 μg/mL (image c), 31.0 μg/mL (image d), 125 μg/mL (image e) and 167 μg/mL (image f). It can be seen that the number of fluorescent particles attached to the biotin-modified glass slide was concentration dependent. Moreover, aggregation of the silica particles was only observed at higher concentrations than 167 μg/mL. Even though the silanization of glass surfaces with trialkoxysilanes is relatively simple and well-known, the silane density is difficult to control and an inherent standard deviation of 22% for thiol groups density on glass slides surfaces prepared by liquid-phase silanization, was reported (Zhao et al., 2004). Based on these experiments we determined that the optimum concentration to attach streptavidin-coated fluorescent particles to the biotinylated glass slides that resulted in an average of 1300 particles per field of view without aggregation was 125 μg/mL. The variability in the number of particles between fields of view on the

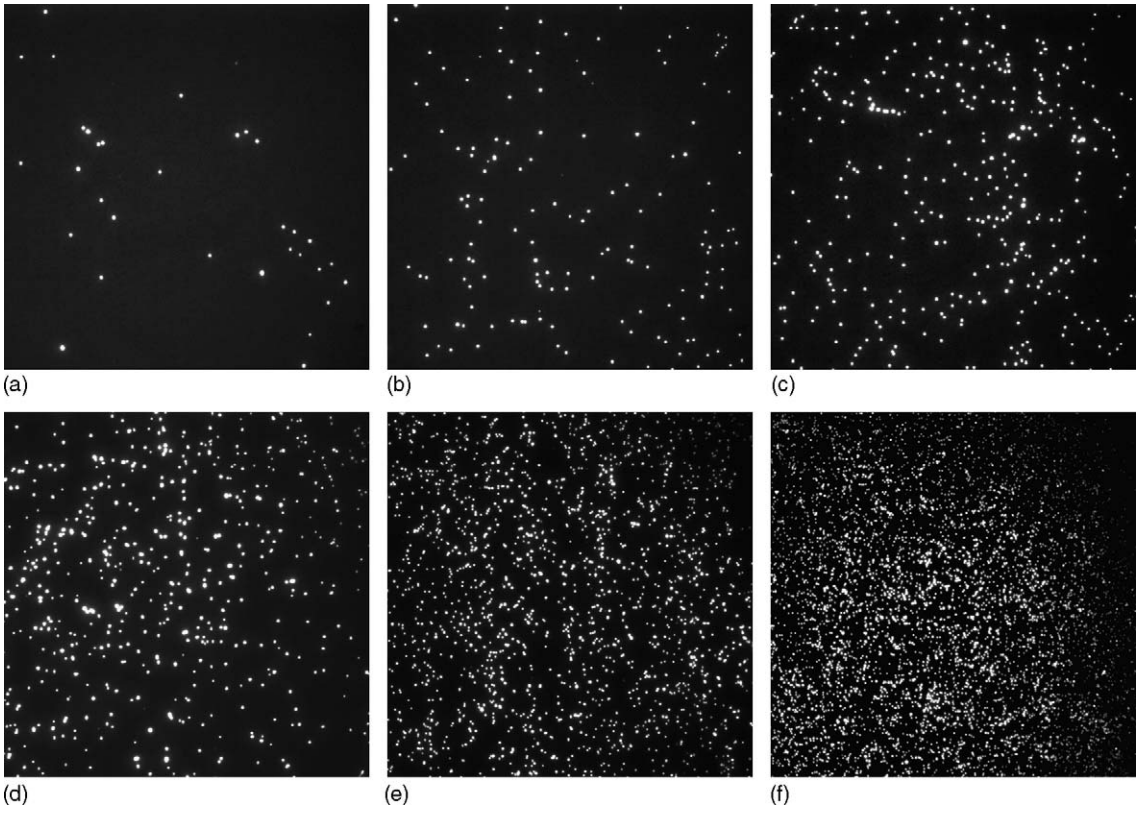

Fig. 3. Digital fluorescence images of streptadivin coated fluorescent silica particles that were immobilized to biotinylated glass slides: (a) a control experiment—125 μg/mL thiol-modified fluorescent silica particles adsorb non-specifically to a biotinylated glass slide. Minimal non-specific adsorption is observed. (b) 6.25 μg/mL, (c) 12.5 μg/mL, (d) 31 μg/mL, (e) 125 μg/mL and (f) 167 μg/mL of streptadivin coated fluorescent silica particles. All glass slides were washed thoroughly with a phosphate buffer at pH 7.4 to remove non-specifically bound particles. The images were taken through a 20× microscope objective using a 460 ± 10 nm band pass excitation filter, a 505 nm dichroic mirror and a 515 nm long pass emission filter.

glass slides was about 20%. To decrease the standard deviation each data point was based on the digital counting of fluorescent particles in 10 randomly selected fields of view. This decreased the standard deviation of our digital counting assays to about 7%. Fluorescent particle suspensions of the optimum concentration (125 μg/mL) were used in the digital assays described in the following section.

3.4. Competitive assay for the detection of the breast cancer marker HER2/neu in solution

We developed a competitive assay based on the inhibition of interactions between anti-HER2/neu coated fluorescent nanoparticles and glass slides modified with HER2/neu to detect free HER2/neu in solution using our digital counting technique. Biotin-labeled anti-HER2/neu molecules were attached to the streptavidin-coated silica particles through streptavidin–biotin interactions. We then prepared HER2/neu-modified glass slides by first silanizing the glass slides with APTMS and then using an EDC coupling reaction to covalently attach anti-HER2/neu to the glass slides. Free HER2/neu molecules competed with the HER2/neu-modified glass slides on binding to the anti-HER2/neu-modified silica particles. It was expected that the number of fluorescent particles attached to the glass slides would decrease with increasing concentrations of free HER2/neu in the analyte solutions. To carry out the competitive assays HER2/neu-modified glass slides were incubated with HER2/neu solutions ranging from 10^{-7} to 0.1 mg/mL in PBS buffer at pH 7.4 for 30 min. The glass slides were washed with phosphate buffer to remove excess HER2/neu. The glass slides were then incubated for 30 min in a solution of anti-HER2/neu-coated particles at 125 μg/mL. The excess of particles was washed out and the slides were dried under a stream of nitrogen prior to digital fluorescence imaging microscopy counting measurements. Fig. 4 describes the dependence of N/N_0 where N and N_0 are the numbers of attached fluorescent particles in the presence (N) and absence (N_0) of HER2/neu on the concentration of free HER2/neu in the analyte solutions. In the absence of HER2/neu N/N_0 was 1 while at HER2/neu concentrations higher than 10 μg/mL N showed a background reading that accounted for non-specific adsorption of fluorescent nanoparticles to the HER2/neu-modified glass slides. The limit of detection of the inhibition assays was as low as 1 ng/mL with a linear range between 1 ng/mL and 10 μg/mL of HER2/neu. The high sensitivity and simplicity of these model assays suggested that fluorescent silica nanoparticles prepared using the simple Stöber method could be used as signal amplifiers in qualitative and quantitative assays through antigen–antibody interactions for the detection of soluble cancer markers.

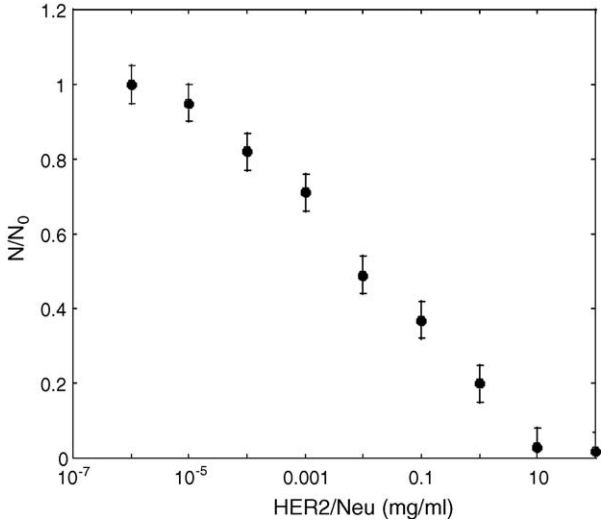

Fig. 4. Competitive immunoassay. Dependence of the number of fluorescent particles immobilized to anti-HER2/neu-modified glass slides on the concentration of free HER2/neu in the analyte solution. Each data point was obtained by averaging the number of attached fluorescent particles in 10 randomly selected fields of view.

4. Summary and conclusions

We have developed a simple method to prepare bright and photostable fluorescent silica nanoparticles of different sizes and narrow size distribution. The method is based on the use of the Stöber method to prepare silica nanoparticles in the presence of the ruthenium diimine complex, $Ru(phen)_3$ to form bright fluorescent particles. It is a one-pot synthetic method that avoids microemulsion formulations and the use of surfactants. The fluorescent particles prepared using the Stöber method showed higher photostability compared to fluorescent particles prepared by dye loading. The ruthenium diimine complex used in this study presents a unique case in which a high encapsulation efficiency of fluorescent dye in silica particles is realized through the use of the Stöber method. Previous attempts to encapsulate organic dyes in these particles encountered difficulties and resulted in poor leaking stability and poor encapsulation efficiency. The high encapsulation efficiency of ruthenium diimine complexes in silica particles is attributed to the unique structure of these transition metal complexes. Since these complexes exhibit high photostability, high emission quantum yield and long fluorescence lifetime they are attractive candidates for the formation dye-containing particles. The ability to form the particles using a one-pot synthesis approach following a standard Stöber-like protocol is highly advantageous since the synthesis could be easily scaled up to form large amounts of dye-containing particles using a simple synthetic approach and relatively low cost. To demonstrate the utility of the highly fluorescent silica nanoparticles in bioassays, we further modified their surface with streptavidin to facilitate their bioconjugation through streptavidin–biotin interactions. As an example, biotin-modified glass slides were used as supports to bind the streptavidin-labeled fluorescent silica particles. The glass slides were analyzed using digital fluorescence imaging microscopy to show the dependence of the number of attached nanoparticles on the concentration of particles in solution. A digital counting competitive immunoassay to detect the breast cancer marker HER2/neu using the new fluorescent nanoparticles as signal amplifiers was performed. The assay was shown to be simple and highly sensitive with a limit of detection of 1 ng/mL and a linear range between 1 ng/mL and 10 μg/mL of HER2/neu.

It should be mentioned that the error bar in the analysis precludes determination of HER2/neu levels that are lower than 1 ng/ml. This is attributed to the manual nature of the assay as performed in our laboratory. With automation and the ability to acquire multiple fields and analyze larger number of particles for a given HER2/neu concentration, further improvement in analytical properties is expected. As is, the assay could easily discriminate between normal serum samples and serum samples spiked with physiologically relevant levels of HER2/neu. In summary, digital counting of nanoparticles as a detection mode in bioanalytical assays is an attractive alternative to detection techniques involving analog fluorescence detection since they could be used for rapid testing of soluble cancer markers in biological fluids. Further assay development particularly automation and the development of high throughput analysis methods are necessary to further improve the assay performance in order to transfer the technology from academic settings to clinical diagnostic laboratories.

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