Colloids and Surfaces B: Biointerfaces 152 (2017) 475–481

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Inherently fluorescent polystyrene microspheres for coating, sensing and cellular imaging

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ARTICLE INFO

Article history:
Received 20 September 2016
Received in revised form
22 November 2016
Accepted 22 December 2016
Available online 4 February 2017

Keywords:
Chloromethylated polystyrene microspheres
Coating
Fluorescence
Fe^{3+}
Cellular imaging

ABSTRACT

Commercially available polystyrene (PS) fluorescent microspheres are widely used in biological field for tracing, in vivo imaging and calibration of flow cytometry, among other applications. However, these particles do suffer from some drawbacks such as the leakage and photobleaching of organic dyes within them. In the present study, inherently fluorescent properties of PS based microspheres have been explored for the first time. Here we find that a simple chloromethylation reaction endows the polystyrene particles with inherent fluorescence without any subsequent conjugation of an external fluorophore. A possible mechanism for fluorescence is elucidated by synthesizing and investigating *p*-ethylbenzyl chloride, a compound with similar structure. Significantly, no photobleaching or leaking issues were observed owing to the stable structure of the microspheres. Chloromethylated PS (CMPS) microspheres can keep their perpetual blue fluorescence even in dry powder state making them attractive as a potential coating material. Furthermore, the chloromethyl groups on CMPS microspheres make them very convenient for further functionalization. Poly(ethylene glycol) (PEG) grafted microspheres showed good biocompatibility and negligible cytotoxicity, and could be used to image intracellular Fe^{3+} due to the selective fluorescence quenching effect of aqueous Fe^{3+} in cytoplasm.

1. Introduction

Fluorescent microspheres are able to luminesce under UV illumination, which provides contrast and visibility of the microspheres relative to the background. Due to this unique property, they have been intensively employed as tracers for water- and air-flow testing. Biologically, fluorescent microspheres are widely used in medical imaging and flow cytometry [1–4]. Conventional methods to prepare fluorescent microspheres include: encapsulation of small molecular dyes or fluorescent nanomaterials [5–8], covalent linking of fluorophores [9,10], copolymerization with dye-functional monomers [2,11,12], and self-assembly [13]. Although entrapment of dyes is a straight forward design, the leaching of these dyes can be pronounced, reaching up to 50% within 48 h [14]. Stability can also be an issue for self-assembled fluorescent nanoparticles [15–18]. Covalent attaching labels and copolymerization with fluorescent monomer could overcome the leakage of dye and provide more stable fluorescence signal, but inevitably, extra chemical reactions often lead to an increase in the complexity and cost. In addition, the copolymerization with fluorescent monomer can lead to the interaction of fluorophores with the polymeric bulk material, changing the photophysical behavior of the dye and inducing aggregation [2]. It has been reported that covalently coupled dyes showed photochemical instability due to the undesirable interaction between the fluorophores and surrounding compounds [19,20]. It is therefore desirable to simplify the system while retaining all the fluorescent properties.

Fluorophore-modified polystyrene microspheres are commercially available (from Polyscience, Dow Chemicals, etc.) in various sizes and colors. In biological field they have proved to be effective tools for diagnosis, in vivo imaging, optical tracking tracing and calibration of flow cytometry, etc. Their non-biodegradability makes them traceable in vitro and in vivo for long periods of time [7]. However, as with any traditional fluorescent microsphere, leakage of the organic dyes can lead to overestimation of the analyte concentration and can also produce a high background for optical microscopy analysis [21]. In addition, hydrophobic aggregation

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http://dx.doi.org/10.1016/j.colsurfb.2016.12.043
0927-7765/

of fluorescent PS microspheres is common in aqueous solution, limiting their practical application in biomedical field.

Herein, we have found that PS microspheres, after chloromethylation [22], exhibit intriguing inherently fluorescent properties without the need to conjugate any external fluorophores. The fluorescent PS microspheres showed excellent stability with no obvious photobleaching or leaking issues after one month of continuous soaking and irradiation. Control experiments were carried out and revealed the mechanism of the luminescence to be the rigid conjugated structure developed by the Friedel-Crafts reaction, which makes the microspheres fluorescent even when dried as a film in air, showing great application potential as a coating material. In addition, the fluorescent PS microspheres showed a sensitive response to Fe^{3+}. After further hydrophilization with PEG, the microspheres showed excellent biocompatibility and was successfully utilized for intracellular Fe^{3+} imaging.

2. Experimental

2.1. Materials and methods

Cross-linked polystyrene microspheres (1–2 μm, crosslinking degree is 20%) were ordered from Wuxi Knowledge & Benefit Sphere Tech. Co. Ltd; chloromethyl methyl ether (CMME, 40%) was ordered from Zhengzhou Wanxiang Chemicals Co. Ltd; poly(ethylene glycol) methyl ether (Mw 5 kDa), 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, ≥95.0%) and fluorescein isothiocyanate (FITC, 95%) were purchased from Sigma-Aldrich Co. Ltd; Rhodamine Phalloidine (Molecular Probes) was ordered from ThermoFisher Scientific Co. Ltd; anhydrous aluminum chloride ($AlCl_3$, AR), tetrabutyl ammonium bromide (TBAB, AR) and sodium hydride (NaH, 60%) were obtained from Sinopharm Chemical Reagent Co. Ltd; metal iron solutions were prepared from KCl, NaCl, $CaCl_2$, $MgCl_2$, $Al(NO_3)_3$, $CuSO_4$, $ZnCl_2$, $NiCl_2$, $CoCl_2$, $AgNO_3$, $CdCl_2$, $MnSO_4$, $HgCl_2$, $BaCl_2$, NH_4NO_3, $CrCl_3$, $SrCl_2$, $Pb(NO_3)_2$, $FeSO_4$, and $FeCl_3$, respectively. Other reagents and inorganic salts were of analytical grade from local sources.

The morphologies and sizes of fluorescent microspheres were characterized by scanning electron microscope (SEM, S-4800, Hitachi, Japan) and transmission electron microscopy (TEM, JEM-1400 plus, JEOL, Japan). UV–vis absorption and fluorescence spectra were recorded on a Shimadzu UV-2450 spectrophotometry and a Hitachi F-2500 fluorescence spectrophotometer, respectively. The fluorescence images and phase contrast bright-field images of particles and cells were recorded on a confocal laser scanning microscope (CLSM, Nikon A1, Japan). The chemical composition changes of PS microspheres before and after modification were determined using KBr pellets on a Nicolet 6700 FT-IR spectrometer (USA). Dynamic light scattering (DLS) analyses were run on a Zetasizer Nano ZS (Malvern, UK). Static water contact angles of microspheres were measured using an optical contact angle measuring device (DSA100, Kruss, Germany) by placing them densely on a slide glass with a double faced adhesive tape. The water was dropped onto the slide glass by a needle at 0.5 μL per drop. At least three measurements were made for each sample.

2.2. Preparation of CMPS microspheres

CMPS microspheres were prepared via Friedel-Crafts alkylation (Scheme 1A). Briefly, dry PS microspheres (5 g, 38.5 mmol) were swollen in CMME (50 mL) for 60 min and then added $AlCl_3$ (1.2 eq. based on PS microspheres) into the mixture. After stirred for 12 h at 50 °C, the microspheres were collected by centrifuging and washed with acetone, HCl solution (4 N), ethanol and deionized water for several times. The chloride content of CMPS microspheres determined by Volhard back titration method was 13.3 wt%.

2.3. Preparation of CMPS-PEG microspheres

PEG was grafted on CMPS microspheres via Williamson reaction (Scheme 1B). Typically, dry CMPS microspheres (0.5 g, 2.1 mmol) were immersed in anhydrous *N*,*N*-dimethylformamide (DMF, 10 mL) in 2-necked flask overnight. A mixture solution containing PEG (3 g, 0.6 mmol), NaH (0.24 g, 6 mmol) and TBAB (0.322 g, 1 mmol) in DMF (50 mL) was introduced into the flask. After stirring 24 h at 65 °C, the microspheres were collected and washed with ethanol and water for several times. The amount of PEG grafted to CMPS microspheres was determined as the difference between initial and remaining amount. The unreacted PEG was quantified by I_2-KI method [23].

2.4. Cell imaging

The HepG2 cells were seeded onto sterile coverslips in a 24-well plate and cultured with DMEM medium containing 10% FBS for 48 h at 37 °C and humidified atmosphere of 5% CO_2. Then the cells were separated from culture medium, washed with PBS buffer and incubated with CMPS-PEG microspheres (10^6 particles mL^{-1}) for 2 h. Parallel experiment was performed to detect the quenching effect of Fe^{3+}, HepG2 cells were cultured with DMEM medium which contained Fe^{3+} (12 mM) for 6 h in advance, then incubated with CMPS-PEG microspheres (10^6 particles mL^{-1}) for 2 h. Subsequently, the cells were separated, fixed by 4% paraformaldehyde and washed with PBS buffer. Followed, the membrane was dyed by Rhodamine Phalloidin (10 μg mL^{-1}) for 20 min, then washed with PBS buffer. And nucleus was stained by DAPI (5 μg mL^{-1}) for 20 min, then washed. At last the fixed cells with 10 μL of 50% glycerol/PBS (v/v) were placed on slide glass covered with coverslip and observed by CLSM.

3. Results and discussion

3.1. Fabrication of inherently fluorescent microspheres

The fluorescent microspheres (chloromethylated PS microspheres, CMPS) were fabricated via Friedel-Crafts alkylation on the surface of crosslinked polystyrene microspheres. In order to improve their biocompatibility and dispersibility in aqueous medium, CMPS microspheres were further grafted with PEG. The reactions were briefly illustrated as Scheme 1. The morphologies of the PS, CMPS and CMPS-PEG microspheres were shown in Fig. 1A–C. All particles were monodisperse and spherical. The average size of microspheres was 1.2 μm as estimated from the SEM images. After chloromethylation and PEGylation, no apparent change in the morphology of the microspheres was observed (see TEM images in Fig. S1).

The physical properties of PS, CMPS and CMPS-PEG microspheres are summarized in Table 1. Although the grafting density of PEG is 48.8 mg g^{-1}, no significant change in the size of the microspheres before and after functionalization was observed. However, the water content of PS microspheres increased from 14.55% to 19.9% and 53.4% after chloromethylation and PEGylation, respectively. Correspondingly, the water contact angle decreased from 119° to 107° and 58°, respectively (Fig. S2), indicating the hydrophobic surface of CMPS microspheres was effectively shielded after PEGylation. The successful surface modification was also showed by FT-IR (Fig. 1D). Compared to the spectrum of PS microspheres, new peaks at 1265 cm^{-1} and 674 cm^{-1} in spectrum of CMPS were observed and ascribed to the bending vibration CH_2Cl and the stretching vibration of C—Cl, respectively. After further