Preparation of polystyrene fluorescent microspheres based on some fluorescent labels

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Five fluorescent labels were synthesized and used in the preparation of polystyrene fluorescent microspheres by the dispersion copolymerization and absorption method. Spectral properties of copolymerization fluorescent microspheres in tetrahydrofuran indicated these individual characteristics of labels should be maintained in the fluorescent microspheres. The differences of the fluorescent spectra between five fluorescent microspheres and their corresponding parent labels in ethanol have been investigated. These fluorescent microspheres were characterized by environmental scanning electron microscopy, laser scanning confocal microscopy and fluorescence spectrophotometry. They exhibited good dispersion and stable and high fluorescence intensity. Furthermore, copolymerization fluorescent microspheres were functionalized with amino groups. This means that a method for the preparation of copolymerization fluorescent microspheres was developed as a platform for the generation of functional fluorescent microspheres for diverse applications.

Introduction

Fluorescent microspheres are widely used in the standarization of fluorescent-based instruments as well as the detection and analysis of biological and synthetic molecules.[1,2] Surface functionalized fluorescent microspheres in particular have received much attention because of their present and foreseen applications in the biomedical field.[3–5] However, the application field of fluorescent microspheres has been limited by their difficult preparation, limited visibility, tendency to clump together and background fluorescence. Thus, a number of fluorescent microspheres designed to overcome these problems have been reported recently.[1,6–11]

Normal fluorescent labels, rhodamine, fluorescein and Nile red, are often used to prepare fluorescent spheres.[12–16] Rhodamine and fluorescein have good biocompatibility, high quantum yields and large extinction coefficients.[17–20] Nile red is well-known as an environment-sensitive fluorescent probe for labelling and sensing biomolecules.[21,22] In addition, micro-size polystyrenes have high active surface areas, and excellent hydrophobicities and affinities, which are widely used in biological medicine and colloidal science.[23,24]

In this paper, we synthesized allyl rhodamine B, allyl Nile red, allyl fluorescein, unsymmetrical rhodafluor and pyrrolidinyl rhodamine S for the preparation of polystyrene-based fluorescent microspheres. Three kinds of copolymerization fluorescent microspheres were prepared by dispersion polymerization.[25] Moreover, unsymmetrical rhodafluor and pyrrolidinyl rhodamine S were incorporated into polystyrene microspheres with sulfonic groups to prepare fluorescent microspheres. Spectral properties of copolymerization fluorescent microspheres in tetrahydrofuran were studied. And the differences of the fluorescent spectra between five fluorescent microspheres and their corresponding parent labels in ethanol had been investigated, respectively. These fluorescent microspheres were observed by environmental scanning electron microscopy, laser scanning confocal microscopey and fluorescence spectrophotometry. Furthermore, the introduction of amino groups to copolymerization fluorescent microspheres was carried out using silane coupling reagents with vinyl, amido and ethoxy by a sol–gel process.

Experimental

^{1}H NMR and ^{13}C NMR spectra were recorded on an INOVA 500 and BRUKER AV400, respectively. High resolution mass spectra (HRMS) were determined on an IonSpec 7.0T FT-ICR mass spectrometer. UV-vis absorption spectra were measured on a computer-controlled Shimadzu UV-2450 spectrometer. Fluorescence spectra were recorded at room temperature on a Cary Eclipser fluorescence spectrometer. The Φ measurement of dyes was performed on an Edinburgh FLS920 spectrometer.

The average diameter and size distribution of microspheres were measured using a Brookhaven Particle Size Analyzer (BI-90 plus). The environmental scanning electron images (ESEMs) were obtained using a XL30 environmental scanning electron microscope. The confocal microscopic images were obtained using an OLYMPUS FV1000 laser scanning confocal microscope. FT-IR spectroscopy was performed on a NICOLET 380 Fourier transform infrared spectrometer.

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All of the chemicals used in this study were of analytical grade or purified according to standard procedures. Two kinds of polystyrene microspheres with diameters of 1.81 ± 0.03 and 2.23 ± 0.02 μm were purchased from Tianjin Baseline ChroTech Research Centre (China).

Synthesis of fluorescent labels

Allyl rhodamine B 1[26]**.** A mixture of rhodamine B (2.40 g, 5.0 mmol), allyl bromide (0.73 g, 6.0 mmol), Na_2CO_3 (2.65 g, 25.0 mmol), hydroquinone and iodine (trace), and dry DMF (50 mL) was heated and stirred at 71 °C in the dark under N_2 for 25 h. Vacuum evaporation of the solvent yielded the crude product. The title compound was isolated by column chromatography on silica (2.00 g, 77.5%) (methanol/dichloromethane = 1 : 40, R_f = 0.2).

^{1}H NMR (500 MHz, CD_3OD): δ 1.31 (t, J = 7.0 Hz, 12H), 3.69 (q, J = 7.0 Hz, 8H), 4.46 (d, J = 8.5 Hz, 2H), 5.05–5.12 (m, 2H), 5.59–5.66 (m, 1H), 7.00 (d, J = 2.5 Hz, 2H), 7.05 (dd, J = 9.5, 2.5 Hz, 2H), 7.11 (d, J = 9.0 Hz, 2H), 7.46 (d, J = 7.5 Hz, 1H), 7.82 (t, J = 8.0 Hz, 1H), 7.89 (t, J = 7.5 Hz, 1H), 8.32 (d, J = 8.0 Hz, 1H).

Allyl fluorescein 2[27]**.** A mixture of fluorescein (2.00 g, 6.0 mmol), allyl bromide (2.42 g, 20.0 mmol), K_2CO_3 (4.97 g, 36.0 mmol), hydroquinone and iodine (trace), and dry DMF (60 mL) was heated and stirred at 71 °C in the dark under N_2 for 25 h. The solvent was removed under reduced pressure. The crude product was recrystallized from carbon tetrachloride, and the resulting product was separated by column chromatography on silica (0.91 g, 40.5%) (dichloromethane, R_f = 0.1).

^{1}H NMR (500 MHz, $CDCl_3$): δ 4.46 (d, J = 6.5 Hz, 2H), 4.69 (d, J = 4.5 Hz, 2H), 5.09–5.12 (m, 2H), 5.37–5.49 (m, 2H), 5.56–5.62 (m, 1H), 6.03–6.09 (m, 1H), 6.72 (d, J = 9.5 Hz, 2H), 6.84 (d, J = 8.0 Hz, 1H), 6.95–7.03 (m, 3H), 7.32–7.34 (m, 1H), 7.69–7.76 (m, 2H), 8.28 (d, J = 7.0 Hz, 1H).

Allyl Nile red 3. Allyl Nile red was synthesized according to the method of Martin-Brown *et al.*[28] in the yield of 19.3%.

^{1}H NMR (500 MHz, DMSO-d_6): δ 1.23 (t, J = 8.5 Hz, 6H), 3.46 (q, J = 7.0 Hz, 4H), 4.74 (d, J = 5.5 Hz, 2H), 5.30 (dd, J = 10.5, 1.5 Hz, 1H), 5.45 (dd, J = 17.5, 1.5 Hz, 1H), 6.06–6.11 (m, 1H), 6.20 (s, 1H), 6.64 (s, 1H), 6.82–6.85 (m, 1H), 7.27 (dd, J = 8.5, 2.5 Hz, 1H), 7.61 (d, J = 9.0 Hz, 1H), 7.95 (s, 1H), 8.03 (d, J = 9.0 Hz, 1H).

Unsymmetrical rhodafluor 6

2-Carboxyl-4′-diethylamino-2′-hydroxy benzophenone ***4***. 3-Diethylamino phenol (3.30 g, 20.0 mmol) and phthalic anhydride (3.00 g, 21.0 mmol) in toluene (20 mL) were refluxed under nitrogen for 4 h. The mixture was cooled to 50–60 °C. 20 mL of 35% aqueous NaOH (*w*/*w*) was added and then heated at 90 °C for 6 h. The resulting mixture was poured into 200 mL of H_2O, acidified with HCl (10.0 mol L^{-1}), and allowed to stand at room temperature for 2 h. The suspension was filtered, and the solid was recrystallized from the mixture of water and methanol, then dried to afford the desired product (4.75 g, 73.1%).

^{1}H NMR (500 MHz, CD_3OD: DMSO-d_6 = 5 : 1): δ 1.15 (t, J = 7.0 Hz, 6H), 3.40 (q, J = 7.0 Hz, 4H), 6.09 (d, J = 2.5 Hz, 1H), 6.15 (d, J = 9.0 Hz, 1H), 6.84 (d, J = 9.5 Hz, 1H), 7.35 (dd, J = 7.5, 1.0 Hz, 1H), 7.58 (t, J = 7.5 Hz, 1H), 7.66 (t, J = 7.5 Hz, 1H), 8.03 (d, J = 8.0 Hz, 1H).

Unsymmetrical rhodafluor ***5***. Compound **4** (0.50 g, 1.6 mmol) was added to a solution of resorcinol (0.20 g, 1.8 mmol) in 10 mL methanesulfonic acid. The resulting suspension was heated under N_2 at 85 °C for 14 h. The mixture was poured into 50 volumes of ice water, and then neutralized with saturated aqueous Na_2CO_3, followed by filtration. The cake was dried to give crude product. The title compound was separated by column chromatography on silica (0.31 g, 73.8%) (methanol/dichloromethane = 1 : 20, R_f = 0.2). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.12 (t, J = 7.2 Hz, 6H), 3.60 (q, J = 7.2 Hz, 4H), 6.31 (d, J = 2.2 Hz, 2H), 6.62 (d, J = 7.4 Hz, 2H), 6.89 (d, J = 2.4 Hz, 1H), 7.11 (d, J = 9.6 Hz, 1H), 7.32–7.35 (m, 1H), 7.73–7.76 (m, 2H), 8.17 (d, J = 7.6 Hz, 1H).

Unsymmetrical rhodafluor ***6***. Thionyl chloride (0.55 mL) was added to a solution of compound **5** (0.60 g, 1.5 mmol) in *n*-butanol (5 mL) at 0 °C. The mixture was stirred for 1 h, and then heated at 70 °C for 7 h. The solvent was evaporated under reduced pressure. The residual material was purified by column chromatography on silica to afford compound **6** (0.62 g, 90.0%) (methanol/dichloromethane = 1 : 80, R_f = 0.3).

^{1}H NMR (500 MHz, CD_3OD): δ 0.72 (t, J = 7.25 Hz, 3H), 0.96–1.04 (m, 2H), 1.18–1.2 (m, 2H), 1.22 (t, J = 7.0 Hz, 6H), 3.46 (q, J = 7.0 Hz, 4H), 3.93 (t, J = 6.5 Hz, 2H), 6.54–6.61 (m, 4H), 6.83–6.86 (m, 2H), 7.24–7.27 (m, 1H), 7.63 (t, J = 7.5 Hz, 1H), 7.68 (t, J = 7.5 Hz, 1H), 8.20 (d, J = 7.5 Hz, 1H).

^{13}C NMR (400 MHz, CD_3OD): δ 184.93, 167.22, 160.95, 158.01, 157.36, 155.44, 135.20, 133.82, 132.21, 132.14, 132.09, 131.68, 131.56, 131.12, 126.49, 115.02, 113.43, 112.92, 105.01, 97.27, 65.52, 46.38, 31.50, 20.20, 14.09, 12.87.

HRMS (ESI) $C_{28}H_{29}O_4N$: calcd. 444.2169; found *m*/*z* 444.2172 $[M]^+$.

Pyrrolidinyl rhodamine S 7

3-Pyrrolidinylphenol was prepared according to the method of Lee *et al.*[29] in the yield of 58.7%.

^{1}H NMR (500 MHz, DMSO-d_6): δ 1.89–1.92 (m, 4H), 3.12–3.16 (m, 4H), 5.91 (t, J = 2.0 Hz, 1H), 5.95–6.02 (m, 2H), 6.88 (t, J = 8.0 Hz, 1H), 8.94 (s, 1H).

The mixture of 3-pyrrolidinylphenol (0.49 g, 3.0 mmol), crushed succinic anhydride (0.35 g, 3.5 mmol) and $ZnCl_2$ (0.47 g, 3.5 mmol) was heated at 160 °C under N_2 for 4 h. After cooling, the solid was pulverized and diluted with 2.5 mL of ethanol. 4.6 mL of $HClO_4$ (60%) and 92 mL of water were added consecutively to the solution, which was kept at 5 °C for 72 h. The precipitate was collected and dried. The crude product was isolated by column chromatography on silica (0.33 g, 55.9%) (methanol/dichloromethane = 1 : 100, R_f = 0.2).

^{1}H NMR (500 MHz, DMSO-d_6): δ 2.02 (t, J = 6.5 Hz, 8H), 2.49 (t, J = 2.0 Hz, 2H), 2.59 (t, J = 7.5 Hz, 2H), 3.54 (t, J = 7.5 Hz, 8H), 6.62 (d, J = 2.5 Hz, 2H), 7.0 (dd, J = 9.5, 2.0 Hz, 2H), 8.05 (d, J = 9.5 Hz, 2H).

^{13}C NMR (400 MHz, DMSO-d_6): δ 172.60, 156.35, 153.96, 129.57, 115.07, 112.26, 96.24, 48.62, 34.81, 24.71.

HRMS (ESI) $C_{24}H_{27}O_3N_2$: calcd. 391.2016; found *m*/*z* 391.2010 $[M]^+$.

Preparation of polystyrene fluorescent microspheres by the dispersion copolymerization[30–35]

An appropriate amount of allyl rhodamine B, or allyl fluorescein or allyl Nile red, styrene (910 mg), 2,2′-azobis(isobutyronitrile) (AIBN, 4.20 mg) and polyvinylpyrrolidone (PVP-K30, 160 mg) were dissolved in ethanol (3.6 mL) and 2-methoxyethanol (2.4 mL) or in ethanol (5.4 mL) and water (1.68 mL). The sealed reaction vessels were immersed into a thermostated water bath and set to rotate for 48 h under N_2 at 70 °C. The solution was poured into ethanol to precipitate and filter fluorescent microspheres. They were washed with ethanol and water until no free dye was removed from microspheres, and then dried under vacuum at 50 °C .

Preparation of fluorescent microspheres with amino groups[36]

The above fluorescent microspheres (200 mg), triethoxyvinylsilane (50 μL) and 2,2′-azobis(isobutyronitrile) (AIBN, 4.00 mg) were added to ethanol (8 mL) and water (1.6 mL). The sealed flask was immersed and set to rotate in a water bath at 70 °C for 24 h. The microspheres were rinsed and centrifuged to remove unreacted reagents and contaminants.

The microspheres were again suspended in the mixture of ethanol (8 mL) and water (1.6 mL) in the presence of ammonia (40 μL) and tetraethoxysilane (50 μL) and stirred for 30 h at room temperature.

After being washed several times, the microspheres were redispersed in ethanol (8 mL) and water (1.6 mL). Ammonia (40 μL) and γ-aminopropyltrioethoxysilane (50 μL) were added to the reaction, which was stirred for 30 h at room temperature. The modified microspheres were rinsed and centrifuged with ethanol, and then dried at 50 °C under vaccum.

Preparation of polystyrene fluorescent microspheres with sulfonic sodium

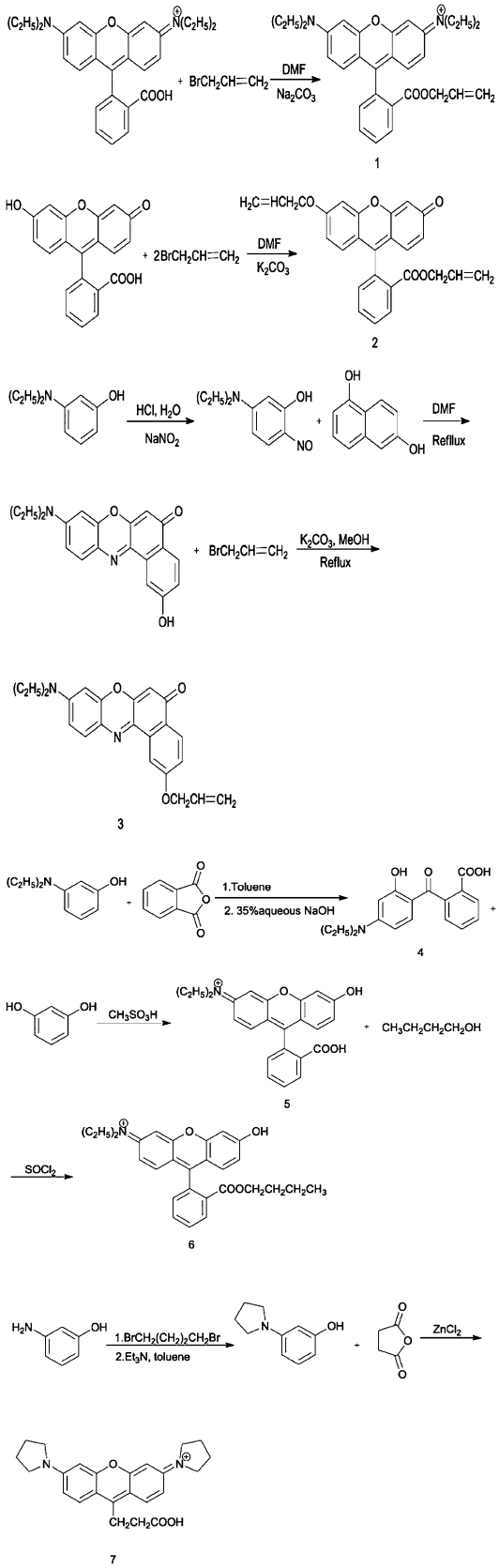
Polystyrene microspheres were sulfonated by sulfuric acid. Polystyrene microspheres with sulfonic groups in 2 mol L^{-1} aqueous NaOH were stirred at room temperature for 2 h and rinsed to neutralization.[37] A solution of compound **6** or **7** dissolved in ethanol was added to the vigorously stirred suspension of the corresponding polystyrene microspheres with sulfonic sodium in ethanol and water ($v : v = 1 : 1$). The resulting suspension was filtered and dialyzed in ethanol to remove any residual dye.

Results and discussion

Syntheses and photophysical properties of fluorescent labels

As shown in Scheme 1, simple chemical reactions yielded the target compounds. Commercially available reagents were used throughout and the overall preparative yields of compounds at the milligram level were satisfactory.

Photophysical properties of five fluorescent labels and their parent dyes are summarized in Table 1. The absorption maximums of **2** are 455 and 473 nm in 0.1 mol L^{-1} NaOH. They are close to the absorption maximums (459 and 483 nm) of 1-[2-(6-methoxy-3-oxo-3*H*-xanthen-9-yl)-benzoyl]-piperidine-4-



Scheme 1 Synthetic scheme for fluorescent labels **1**, **2**, **3**, **6**, **7**.

Table 1 Photophysical properties of five labels and their parent dyes

	1	RhB	**2**	Flu	**3**	Nile red	**6**	**7**
ε/mol cm^{-1} L $\times$ 10^4	6.85	7.85	2.54	8.23	1.60	3.40	3.52	2.39
λ_{ab}/nm	555	542	455[d]	489	543	542	525[d]	548
			473				490	
λ_{ex}/nm	500	500	455	455	543	543	500	500
λ_{em}/nm	586	579	520	534	629	627	553	573
[e]FWHM/nm	33	32	80	39	57	61	35	30
Φ (%)	37.4[a]	50.2[a]	41.8[b]	72.9[b]	46.7[c]	35.9[c]	80.8[c]	83.5[c]

[a] **1**, rhodamine B, **6** and **7** were excited at 500 nm in ethanol. [b] **2** and fluorescein were excited at 455 nm in 0.1 mol L^{-1} NaOH. [c] **3** and Nile red were excited at 543 nm in ethanol. [d] $\lambda_{ab}{}^{max}$ nm^{-1} which represents λ_{ab} of the maximal absorption value. Φ denotes the fluorescent quantum yield. [e] FWHM denotes full width at half-maximum.

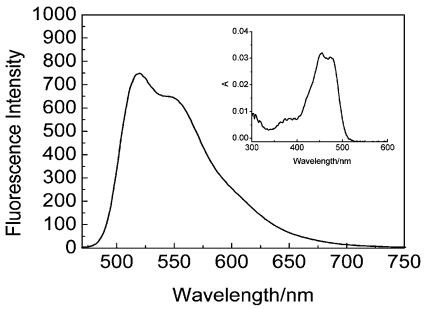


Fig. 1 Fluorescence and UV spectra of **2** in 0.1 mol L^{-1} NaOH (λ_{ex} = 455 nm).

carboxylic acid reported by Gao *et al.*[38] In Fig. 1, the inset shows the UV spectrum of **2**. The broadness of its UV spectrum matches the broadness of its emission spectrum. In addition, the absorption maximums of **6** are 490 and 525 nm. Following comparisons with their parent dyes, the fluorescent spectra of **1**, **2** and **3** produce red shifts with the attachment of the allyl substituent.

The Φ measurement of dyes was performed on an Edinburgh FLS920 spectrometer with an integrating-sphere attachment under excitation of 500, 455 and 543 nm, respectively.[39] The quantum yields of **6** and **7** are higher than those of **1** and rhodamine B under excitation of 500 nm in ethanol. The quantum yield of **2** is lower than that of fluorescein under excitation of 455 nm in 0.1 mol L^{-1} NaOH. The quantum yield of **3** is higher than that of Nile red under excitation of 543 nm in ethanol.

Spectral properties of fluorescent microspheres

The chromophore contents of a1, b2 and c3 were determined by spectroscopy of tetrahydrofuran solutions of the microspheres. The maximum-absorption coefficients of the corresponding **1**, **2** and **3** were used as references in the same solvent. However, d6 and e7 cannot be dissolved in most organic solvents, such as tetrahydrofuran and trichloromethane, because of the large number of sulfonic groups of microspheres. Therefore, a certain amount of **6** or **7** was added to the suspension of 100 mg of the corresponding polystyrene microspheres with sulfonic sodium in ethanol and water ($v : v = 1 : 1$). And the resulting suspension was filtered and dialyzed in ethanol to remove any residual dye. Then the filtrate and dialysate were diluted to 50 mL with the corresponding solvent, respectively. The amount of residual dye was determined, using as references the maximum-absorption coefficients of the corresponding **6** and **7** in the corresponding solvent. The chromophore contents of a1, b2, c3, d6 and e7 are listed in Table 2. The a1, b2 and c3 were obtained by copolymerization between dye and styrene, while d6 and e7 depend on physical action to the absorbing dye. So contents of **1**, **2** and **3** are much lower than those of **6** and **7** in microspheres.

Table 2 The chromophore contents of fluorescent microspheres[a]

	a1	b2	c3	d6	e7
(% *w*/*w*)	2.377 $\times$ 10^{-3}	1.541 $\times$ 10^{-2}	8.498 $\times$ 10^{-3}	1.214	1.458

[a] a1, b2, c3, d6, e7 denote fluorescent microspheres of **1**, **2**, **3**, **6**, **7**, respectively.

The absorption and fluorescent properties of **1**, **2**, **3**, a1, b2, c3 and the corresponding equimolar mixtures of **1**, **2**, **3** and PS in tetrahydrofuran are listed in Table 3, respectively.[26,40,41] Obviously, the absorption and fluorescent spectra of the corresponding monomers and corresponding equimolar mixtures have very similar features as those of the fluorescent microspheres in tetrahydrofuran. This indicates that these individual characteristics of **1**, **2** and **3** should be maintained in the fluorescent microspheres. It can also be suggested that spectral properties of **1**, **2** and **3** in tetrahydrofuran are not affected by the aromatic environment, or the rigid nature of the PS matrix.[42,43]

The comparisions of the emission values of parent labels and five microspheres dispersed in ethanol are listed in Table 4. Undoubtedly, the spectral emissions of labels bonded or incorporated into microspheres were not greatly changed in comparison with those of the labels.

Table 3 λ_{ab}/nm and λ_{em}/nm of **1**, **2** and **3**, fluorescent microspheres and mixtures of **1**, **2** or **3** and PS in THF[a]

	1	**2**	**3**	a1	b2	c3	M1	M2	M3
λ_{ab}	558	455	524	558	455	524	559	455	524
		432			430			432	
λ_{ex}	558	455	524	558	455	524	558	455	524
λ_{em}	580	525	595	580	527	593	580	526	597

[a] a1, b2, c3 denote fluorescent microspheres of **1**, **2**, **3**, respectively. M1, M2, and M3 are the equimolar mixture model systems containing **1**, **2**, **3** and PS corresponding to a1, b2 and c3, respectively.

Table 4 Comparison of λ_{em}/nm of parent labels and five microspheres[a]

	1	a1	**2**	b2	**3**	c3	**6**	d6	**7**	e7
λ_{ab}	555		458 488		543		525		548	
λ_{ex}	555	555	458	458	543	543	525	525	548	548
λ_{em}	578	595	515	555	629	606	546	560	566	586
$\Delta\lambda$	17		40		23		14		20	

[a] a1, b2, c3, d6, e7 denote fluorescent microspheres of **1**, **2**, **3**, **6**, **7**, respectively. Labels (10^{-7} mol L^{-1} in ethanol) and fluorescent microspheres (3% *w*/*w* dispersed in ethanol).

Table 5 λ_{ab}/nm and λ_{em}/nm of **1**, **2** and **3** in ethanol and toluene

	Ethanol		Toluene	
Dyes	λ_{ab}	λ_{em}[a]	λ_{ab}	λ_{em}[a]
1	555	578	564	590
2	458[b] 488	515	460[b] 436	533
3	543	629	523	595

[a] Excited at the absorption maxima of **1**, **2** and **3** in ethanol. [b] λ_{ab}^{max}/nm which represents λ_{ab} of the maximal absorption value.

As shown in Fig. 2, the fluorescent spectra of a1, b2, c3, d6 and e7 produced different shifts in comparison with those of their parent labels. In order to explain the shifts of the fluorescent spectra of a1, b2 and c3, **1**, **2** and **3** were dissolved in toluene, which has a similar chemical composition to polystyrene, but is a liquid. The absorption and fluorescence spectra of **1**, **2** and **3** in ethanol and toluene are tabulated in Table 5 and shown in Fig. 2.

From Tables 4 and 5, the fluorescent spectra of **1** in toluene and a1 in ethanol have a similar extent of red shift corresponding to **1** in ethanol. This demonstrates that the shift of fluorescent spectrum of a1 in ethanol is mainly due to its aromatic environment. The fluorescent spectra of **2** in toluene and b2 in ethanol have a different extent of red shift corresponding to **2** in ethanol. This shows that the change of the fluorescence spectra of

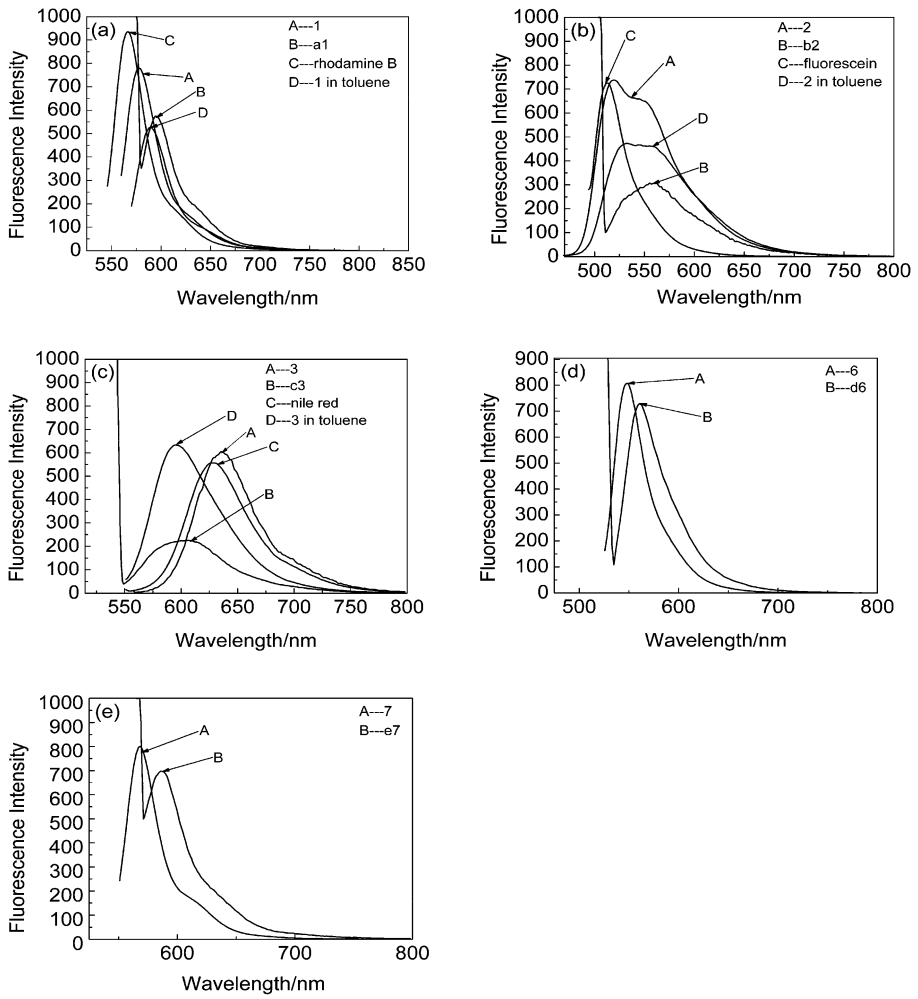


Fig. 2 The emission spectra of labels in ethanol and toluene (**1**, **2** and **3**) and corresponding microspheres dispersed in ethanol (a) **1**, a1, rhodamine B were excited at 555 nm in ethanol. **1** was excited at 555 nm in toluene. (b) **2**, b2, fluorescein were excited at 458 nm in ethanol. **2** was excited at 458 nm in toluene. (c) **3**, c3, Nile red were excited at 543 nm in ethanol. **3** was excited at 543 nm in toluene. (d) **6**, d6 were excited at 525 nm in ethanol. (e) **7**, e7 were excited at 548 nm in ethanol. a1, b2, c3, d6, e7 denote fluorescent microspheres of **1**, **2**, **3**, **6**, **7**, respectively. Labels (10^{-7} mol L^{-1} in ethanol) and fluorescent microspheres (3% *w*/*w* in ethanol).

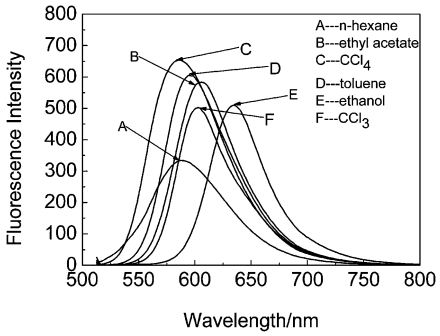


Fig. 3 The emission spectra of **3** in different solvents (λ_{ex} = 510 nm).

b2 in ethanol is due to both the aromatic environment and the rigid nature of the PS matrix. In addition, the spectral shift of **3** occurs when dissolved in different solvents[22] as in Fig. 3 or encaplused in hydrophobic microspheres as described by Haugland *et al.*[1] The fluorescent spectra of **3** in toluene and c3 in ethanol have a different extent of blue shift corresponding to **3** in ethanol. This indicates that the fluorescent spectrum shift of c3 is attributed to a hydrophobic environment, aromatic environment and the rigid nature of the PS matrix.

Table 6 The average diameter and size distribution of microspheres (in μm)[a]

	a1	b2	c3	d6	Blank (d6)	e7	Blank (e7)
Mean	2.553	2.236	2.218	1.806	1.809	2.228	2.227
D	0.010	0.047	0.012	0.029	0.031	0.013	0.015

[a] a1, b2, c3, d6, e7 denote fluorescent microspheres of **1**, **2**, **3**, **6**, **7**, respectively. Mean denotes average diameter. *D* denotes polydispersity index. Blank (d6) and Blank (e7) denote blank microspheres of d6 and e7 with sulfonic groups, respectively.

Finally, the fluorescent spectra of d6 and e7 offered red shifts compared with those of **6** and **7** in ethanol, which are mainly due to the hydrophobic environment of microspheres and the electrostatic interactions between **6** or **7** and the sulfonic sodium of microspheres.

Preparation of copolymerization fluorescent polystyrene microspheres and fluorescent microspheres with amino groups

The average diameter and size distribution of microspheres are given in Table 6.

It is found during the preparation of a1 that the size distribution of microspheres was narrow when the ratio of **1** to styrene is below 3 : 455, otherwise, the size distribution was wide as in Fig. 4 and 5. It is highly probable that steric hindrance of the rhodamine group and self-resisting polymerization of rhodamine monomers prevent chain growth,[26,44] which influences the uniformity of the microspheres. This result has also confirmed that the monomer of **1** had indeed been copolymerized with styrene in spite of its low proportion. In addition, a1 with the average diameter of 2.553 μm was prepared in the mixture of ethanol and 2-methoxyethanol as in Fig. 4 (a). While the microspheres of **1** with an average diameter of 1.128 μm were prepared in aqueous ethanol as in Fig. 4 (b). Moreover, **1** does not significantly change the net surface charge and hydrophobic parking area of polystyrene microspheres owing to the small amount of **1**.

The same phenomena occurred in the preparation of b2 and c3. The ratio of **2** to styrene was only 1 : 910 to maintain uniformity of the microspheres as in Fig. 6. However, b2 still has a wider size distribution than those of the other two copolymerization microspheres, which is because of the two allyl groups of **2** being involved in copolymerization. Only by keeping the ratio of **3** to styrene as 1 : 1820 can the fluorescent microspheres have good fluorescent intensity as shown in Fig. 7.

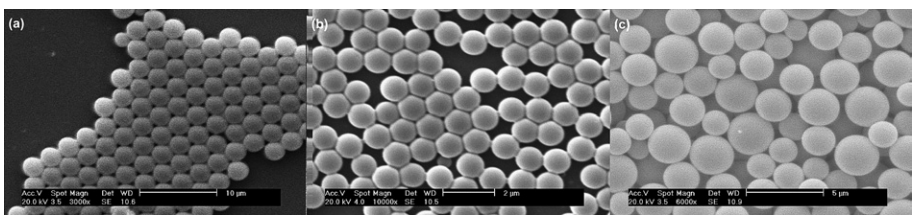


Fig. 4 ESEM images of fluorescent polystyrene microspheres of **1**. (a) The ratio of **1** to styrene is below 3 : 455 (3000×) (fluorescent microspheres with an average diameter of 2.553 μm); (b) the ratio of **1** to styrene is below 3 : 455 (10 000×) (fluorescent microspheres with an average diameter of 1.128 μm); (c) the ratio of 1 to styrene is above 3 : 455 (6000×).

Fig. 5 Confocal microscopic images of fluorescent polystyrene microspheres of **1** (600×). (a) The fluorescent images; (b) their corresponding transmission images (c) the overlays of fluorescent and transmission images. Confocal microscopy images were obtained with laser excitation at 555 nm.

Fig. 6 Confocal microscopic images of fluorescent polystyrene microspheres of **2** (600×). (a) The fluorescent images; (b) their corresponding transmission images; (c) the overlays of fluorescent and transmission images. Confocal microscopy images were obtained with laser excitation at 455 nm.

Fig. 7 Confocal microscopic images of fluorescent polystyrene microspheres of **3** (600×). (a) The fluorescent images; (b) their corresponding transmission images; (c) the overlays of fluorescent and transmission images. Confocal microscopy images were obtained with laser excitation at 543 nm.

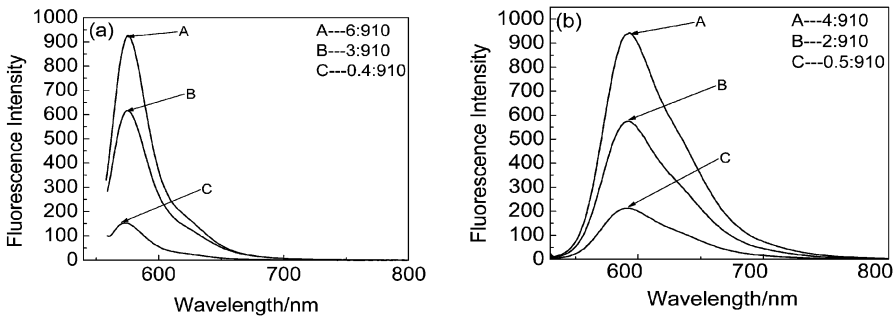


Fig. 8 The emission spectra of the different ratio of dye to styrene in THF. (a) The emission spectra of a1 ($\lambda_{ex} = 558$ nm); (b) the emission spectra of c3 ($\lambda_{ex} = 524$ nm).

As shown in Fig. 8, the fluorescent intensity of a1 and c3 was in proportion to the ratio of **1** or **3** to styrene. There are no fluorescence self quenching labels because of copolymerized with styrene in low proportions.

In order to overcome the problem of no hydroxy group on polystyrene microspheres, silanol groups were firstly attached to the surface of fluorescent polystyrene microspheres by reacting with triethoxyvinylsilane and tetraethoxysilane, respectively. Amino groups were then introduced by reacting with γ-aminopropyltrioethoxysilane, which was confirmed by the IR. In the range of 3448–3376 cm^{-1}, the absorption peak, which was the characteristic one of amino groups, was weak and broad.

The amount of amino groups was determined by the conductometric titration of the anti-titrimetric method due to the amino groups being weak bases. As described in Fig. 9, the amount of amino groups attached on the surface of microspheres was about 105 μmol g^{-1}.

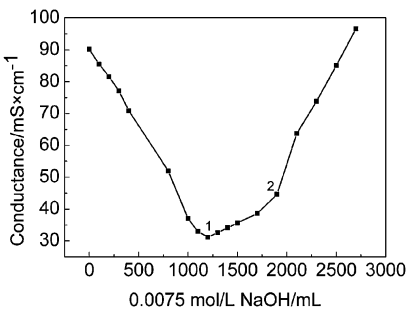


Fig. 9 Conductometric titration curve of fluorescent microspheres of allyl rhodamine B coating with amino groups.

Preparation of absorbing fluorescent microspheres

From Table 6, the average diameter and size distribution of d6 and e7 are in line with those of their corresponding blank microspheres with sulfonic groups. This indicated that absorbing dye had little effect on uniformity of microspheres.

The amount of sulfonic groups of microspheres was about 114 μmol g^{-1} determined by conductometric titration. There are two actions involved in the absorption between microspheres and labels, one is the hydrophobic action, the other is the ionic action. The microspheres with sulfonic sodium have an excellent

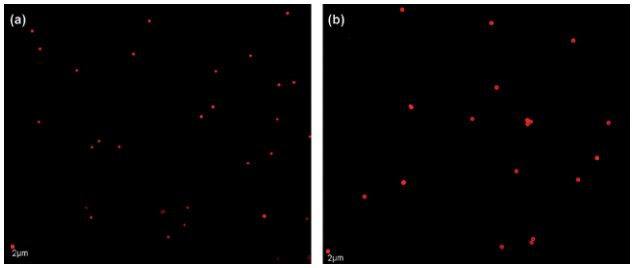


Fig. 10 Confocal microscopic images of fluorescent polystyrene microspheres of **6** and **7** (600×). (a) The fluorescent images of **6** (confocal microscopy images were obtained with laser excitation at 525 nm); (b) the fluorescent images of **7** (confocal microscopy images were obtained with laser excitation at 548 nm).

surface specificity for absorbing lipophilic labels **6** and **7** with positive charges.[45] However, there are still absorption capacities between microspheres and labels. And there is fluorescence self quenching with the increased amount of dye.

The confocal laser scanning micrographs of d6 and e7 in Fig. 10 show that they have good dispersion and photostability as copolymerization fluorescent microspheres. Absorbing fluorescent microspheres are a good choice in some applications when it is inconvenient to prepare copolymerization fluorescent microspheres.

Conclusions

Five fluorescent labels were obtained *via* a simple synthetic pathway. Three kinds of polystyrene-based copolymerization fluorescent microspheres were prepared by dispersion copolymerization. Another two polystyrene fluorescent microspheres with sulfonic groups were produced by absorption of cationic labels through hydrophobic and ionic action. These fluorescent microspheres exhibited good dispersion and stable and high fluorescence intensities. Also, there are no dyes leaching out of these fluorescent microspheres. Amino groups were introduced on the surface of copolymerization fluorescent microspheres by a sol–gel process. Of course, more reactive groups, such as carboxylates, aldehydes, hydroxys, or epoxy groups can be attached on the surface of copolymerization fluorescent microspheres. These functionalized fluorescent microspheres will have good potential for use in various applications, such as biomedical analysis, multitarget detection systems and *in vitro* and *in vivo* imaging. We believe that the method for the preparation of copolymerization fluorescent microspheres will offer a platform for the generation of functional fluorescent microspheres for wide applications.

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