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The preparation of novel AIE fluorescent microspheres by dispersion polymerization

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ABSTRACT

An approach to prepare monodisperse polystyrene microspheres with aggregation-induced emission (AIE) characteristics has been developed which shows promising applications in fluorescence-encoding. The micron-sized, monodisperse polystyrene microspheres with AIE molecules were perfectly synthesized by two-stage dispersion polymerization. Fluorescent AIE monomer was synthesized by Suzuki reaction, confirmed by nuclear magnetic resonance (NMR). These AIE fluorogens (AIEgens) exhibited unique properties such as bright green emission in solid state and increased emission in tetrahydrofuran (THF) solution with the increase of water content. The influence of the AIE molecules concentration to microspheres synthesis was well investigated. The reaction conditions were optimized to obtain the functional polystyrene microspheres with a size distribution around 3%. The novel microspheres were characterized by scanning electron microscopy (SEM), confocal fluorescence microscope and flow cytometry. According to these results, two-stage dispersion polymerization was proved to be an efficient pathway for the preparation of AIE fluorescent and functionalized microspheres, which could be used in many biomedical industries.

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1. Introduction

High-throughput suspension array technology [1] has been widely used in immunoanalysis, nucleic acid detection, disease diagnosis, drug screening and other fields due to its advantages including simple operation, fast acquisition speed and low sample required. Among them, fluorescent microspheres [2] are the key material to distinguish different signals in multiple analysis. In order to meet the practical application, it is significant to develop a more reliable, low cost fluorescent microsphere encoding strategy with larger coding capacity. Organic fluorophores [3] and Quantum dots(QDs) [4–6] are the most commonly used materials for preparing fluorescent encoded microspheres. Quantum dots have a wide excitation spectrum and a narrow emission spectrum, but the synthesis of quantum dots is difficult and expensive. So far, most liquid chip systems [7] reported in the literature used organic dye encoded microspheres. Dye-encoded microspheres [8] could be prepared by copolymerization, covalent bonding, adsorption and swelling of dyes. Solvent swelling method is to swell dye molecules into microspheres by organic solvent, but the dye maybe let out from dye-encoded microspheres. The copolymerization of monomer and dyes avoids the fluorescence leakage of the product, but requires the synthesis of special monomers.

Conventional fluorescent materials have excellent fluorescence associated phenomena in dilute solutions [9], but in practice fluorescent materials are often used in aggregation state. Aggregation-caused quenching (ACQ [10,11]) often appears in those conventional fluorescent materials [12], because ACQ molecules are usually stable condensed ring compounds with planar structure and they are difficult to carry out intramolecular movement like hexaphenylsilole (HPS [13]) molecules in the dispersed state. Thus, the energy needs to be consumed through the fluorescent radiation pathway, so strong fluorescence is produced during the dispersion. In this process, the outermost electron of the electron donor is transferred to the fluorophore vacant orbital [14,15]. One of the most famous ACQ materials is QDs, which are widely used in many important fields, such as

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photoelectric, photovoltaic, biological, medical and so on [9]. But it has two problems: firstly, QDs have many disadvantages such as limited types, complex synthesis and poor stability. Secondly, aggregation of QDs leads to ACQ. For example, when QDs encoded microspheres are suspended in water, they form non-fluorescent aggregates after they lose their coated surfactants. When the polymer chain is closely aggregated in the water medium, the interaction between the molecular chains is strengthened, which leads to the decrease or even completely disappear of fluorescence. Other conventional organic fluorescent materials are small molecules, and the ACQ problem is also very serious. For example, carbazole is a synthetic chromophore with a fluorescent efficiency of 100% at very dilute concentration [16]. But when the concentration is increased to about 10%, the molecules aggregated and the quantum yield dropped to 0%. As the medium of biological systems is water, many organic dyes naturally accumulate in the biological application. Fortunately, the ACQ problem could been solved by Prof. Tang who reported the first AIEgen in 2001, and a large number of AIEgens materials have been developed over the past 20 years [17]. AIE is an unusual photophysical phenomenon that some luminogens show no or weak fluorescence in solution state but high fluorescence in the aggregated and solid state. AIE materials usually have a structure similar to propeller or rotor [18]. These structures are not conducive to the formation of intermolecular π-π interactions in the aggregated state, thus weakening the main pathway of nonradioactive decay in the aggregated state, because of the restriction of intramolecular rotations (RIR) [19–22]. The non-fluorescence of silole in solution is attributed to the active intramolecular rotations of multiple phenyl rings on its periphery, while the strong emission in the solid state is caused by the restriction of intramolecular rotations of those phenyl rings. Despite AIE materials have been applied to many aspects of biological materials, the research on AIE encoded polystyrene microspheres are still poor.

The polystyrene microspheres are widely used as carriers for functional microspheres owing to their excellent chemical stability, easy preparation and functional modification. Therefore, we wondered if there was any possibility of the monodisperse AIE-active conjugated polymer microspheres being obtained via dispersion polymerization. In this paper, we reported a novel AIE monomer with green fluorescence and used two-stage dispersion polymerization [23,24] to copolymerize the AIEgens and styrene, and prepared 3 μm monodisperse microspheres [25] with different content of AIEgens, which were well characterized. In order to evaluate the potential of these polystyrene microspheres in high-throughput array, the fluorescence flow cytometry test was also carried out.

2. Materials and methods

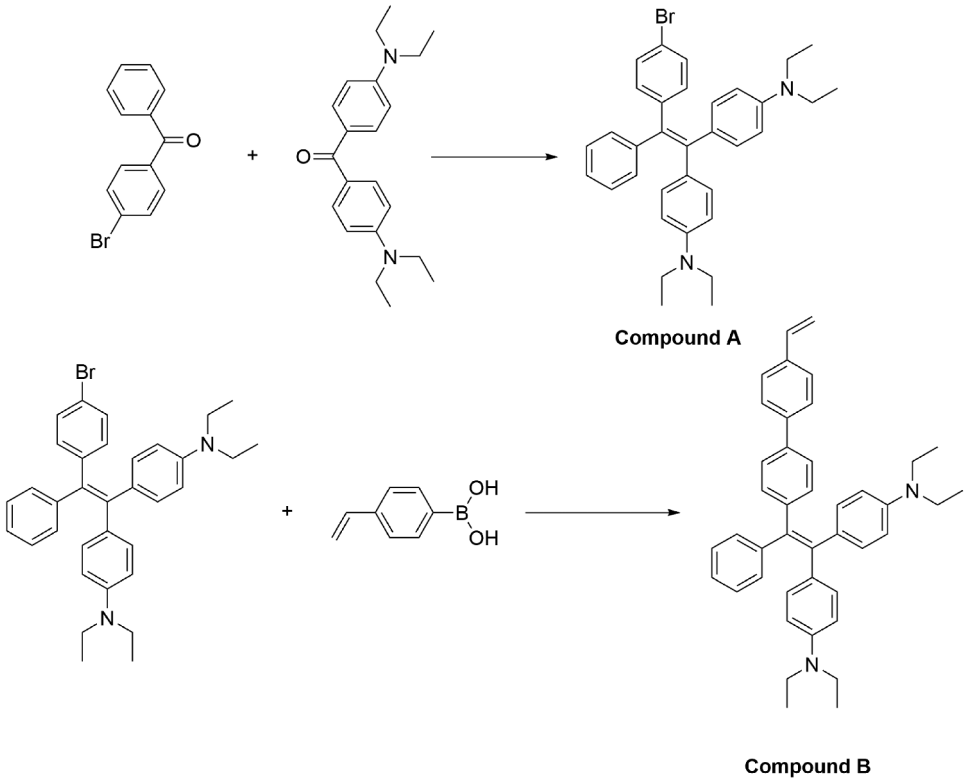
2.1 Reagents

$Pd(PPh_3)_4$,a4-Bromobenzophenone and (4-vinylphenyl) boronic acid were purchased from Leyan Co. Azobisisobutyronitrile (AIBN), 4-vinylbenzyl chloride, Titanium tetrachloride and Pyridine (95%) were obtained from J&K Co. Calcium hydroxide, Acetonitrile (95%), dichioromethane (DCM, ≥99.0%)), THF (≥99.0%), potassium carbonate (K_2CO_3, 99%) and Sodium hydroxide (NaOH) were got from Sinopharm Co. Triton X-305, Polyvidone (PVP, average Mw ~55,000), Styrene (≥99.0%), undecylenic acid (≥96%) and divinylbenzene (DVB) were available from Sigma-Aldrich Co. Anhydrous sodium sulfate, Ethanol (≥99.0%) and Methanol (≥99.0%) were purchased from General-Reagent Co. Sodium dodecyl sulfate (SDS) were obtained from Aladdin Co. Among them, AIBN purified by crystallization before use. All the other reagents were used without purification.

2.2. Synthesis of AIE fluorescent materials

2.2.1. Synthesis of compound A

The synthesis of compound A was according to reference [26]. Zinc dust (0.315 g, 4.84 mmol) and dry THF (20 mL) was added into a 250-mL two-necked round-bottomed flask. The flask was degassed and flushed with dry nitrogen three times, after which THF (20 mL) was injected. The mixture was placed in an ice-salt bath, and then titanium tetrachloride (0.36 mL, 3.0 mmol) was added slowly. The mixture was allowed to warm to temperature to and kept stirring for 0.5 h, after which the reaction mixture was refluxed at 74°C for 2 h. The mixture was then cooled to 0°C again, treated with 0.05 mL of pyridine, and stirred for 10 min. Then, a THF solution (20 mL) of 4-Bromobenzophenone (0.216 g, 0.83 mmol) and 4,4″-Bis(diethylamino) benzophenone (0.292 g, 0.90 mmol) was added slowly. After refluxing overnight, the reaction was quenched with a 10% potassium carbonate aqueous solution and extracted with DCM. The organic layer was washed with brine and dried over anhydrous sodium sulfate. After filtration and solvent evaporation; the residue was purified by silica gel column chromatography, using petroleum ether/ethyl acetate (15:1 by volume) as eluent. The final product was obtained as a green solid in 41.6% yield. ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ = 7.50–7.44 (d, 1H), 7.39–7.34 (d, 1H), 7.31–7.26 (d,



Scheme 1. Synthetic route to Compound A and B.

1H), 7.14–6.75 (m, 13 H), 6.72–6.60 (m, 1H), 6.40–6.24 (m, 4 H), 5.73–5.64 (m, 1H), 5.23–5.12 (m, 1H), 3.32–3.09 (m, 8 H), 1.10–0.97 (m, 12 H).

2.2.2. Synthesis of compound B

Compound A (0.165 g, 0.3 mmol), 4-Vinylbenzeneboronic acid (0.5 g, 0.66 mmol), and $Pd(PPh_3)_4$ (15.2 mg, 0.5%) were added to a 100-mL two-necked round-bottomed flask. The flask was evacuated under vacuum and flushed with dry nitrogen three times. THF (10 mL) and potassium carbonate solution (2 M, 1 mL) were injected into the flask and the mixture was refluxed overnight. The solution was poured into water and extracted with DCM. The organic layer was washed with brine and dried over sodium sulfate. After filtration and solvent evaporation, the residue was purified by silica gel column chromatography, using petroleum ether as eluent. The final product was obtained as a green solid in 50.6% yield. ^{1}H NMR (400 MHz, $CDCl_3$, ppm): δ7.60–7.48 (d, 2 H), 7.47–7.38 (d, 2 H), 7.38–7.32 (d, 2 H), 7.20–6.79 (m, 11 H), 6.78–6.67 (m, 1H), 6.51–6.30 (s, 4 H), 5.82–5.67 (m, 1H), 5.33–5.17 (m, 1H), 3.43–3.09 (m, 8 H), 1.15–1.06 (m, 13 H).

2.3. Synthesis of AIE fluorescent microspheres by two-stage dispersion polymerization

We fabricated AIE fluorescent microspheres using two-stage dispersion polymerization procedure. The stabilizer (PVP 55000) 1.0 g, the co-stabilizer (Triton X-305) 0.35 g, initiator (AIBN) 0.25 g and half of the 6.25 g styrene monomer and 18.75 g ethanol were added to a 250 mL three-necked reaction flask equipped with a gas inlet, overhead stirrer and rubber septum. After a homogeneous solution formed at room temperature, the solution was deoxygenated by bubbling nitrogen gas at room temperature for at least 30 min. Then, the flask was placed in a 70°C oil bath and stirred mechanically at 120 rpm. The AIE material was dissolved in the remaining styrene and ethanol at 60°C under nitrogen. After the fluorescent dye was dissolved and the polymerization reaction has run for 1 h, the hot styrene-dye solution was added into the reaction flask. The reaction was continued for 24 h under continuous flow of nitrogen with overhead stirring. The precipitated polymer in the reaction medium was washed with 200 ml × 4 of ethanol and separated by centrifuge. The polymer was dried under vacuum at 50°C for 24 h.

2.4. Characterization

^{1}H-NMR spectrum was examined on a Bruker 400 MHz instrument, chloroform-d ($CDCl_3$) as solvent. Scanning electron microscopy (SEM) was conducted on a field emission scanning electron microscopy (e Quanta 400 FEG). First, the samples were dispersed in ethanol and then dropped

appropriate sample emulsion on tin foil. When the solvents were completely evaporated, the sample was stick to the sample table and then coated with gold. The micrographs were acquired at an accelerating voltage of 15.0 kV. Microspheres size analysis was based on the SEM image. Generally, the diameters of 100 microspheres were selected to obtain the average diameters (dn) and the coefficient of variation (CV). Fluorescence spectra and solid absolute quantum yield were recorded using a Hitachi F-4600 fluorescence spectrophotometer. UV/Vis spectra were recorded on a Hitachi U-3900 H spectrophotometer.

Flow cytometric analysis was performed on a CytoFLEX Flow Cytometer (BECKMAN COULTER) with a 405 nm excitation laser. The AIEgens fluorescence signal was measured using standard optical filters (Blue laser: 525/40) for the decoding of the fluorescence-encoded beads. Median fluorescence intensity was reported by analyzing 10000 gated beads for each bead set by CytExpert Software.

Confocal fluorescence micrograph was performed on Laser scanning Confocal Microscopy (Leica TCS SP5). The AIEgens fluorescence signal was measured using 400 nm excitation light and observed on 63X oil immersion lens.

3. Result and discussion

3.1. Synthesis and characterization of AIEgens

In this study, we developed a novel AIE monomer with green fluorescence. The compound had good solubility in common organic solvents such as THF, chloroform, and toluene, but was insoluble in polar solvents such as water and methanol. This AIEgen contains a vinyl group which could be used in the following copolymerization for preparation of polystyrene microspheres. The compound B shows fascinating AIE features and has green fluorescence under UV. The compound A and B were characterized by ^{1}H NMR. From Figure 1, we can clearly see appearance of a new set of signals at ~5.0 to 6.0 ppm, which was assigned to the vinyl protons, suggesting the successful synthesis of AIE monomer.

We also investigated the photophysical properties of Compound B. The fluorescence (FL) spectra and UV-vis spectra are shown in Figure 2. Compound B exhibited an emission maximum at 524 nm in dilute THF solution and showed a slight red shift after addition of water. The FL intensity increased with increase in the water fraction (f_w). Compound B showed a strong FL intensity (λ_{abs} of 548 nm) at f_w = 90% due to the formation of aggregates, which was the typical aggregation-enhanced emission (AEE) property of AIE material. The existence of multiple π – π and C – H···π interactions between adjacent molecules in the aggregates could contribute to the red-shifted emission of compound B in aggregates (Figure 2(a)). With the increase of the water content, the degree of red shift became greater, which is also resulted from the degree of AIE monomers aggregation. The transparent solution of Compound B shows a comparable absorption maximum at about 325 nm (Figure 2(b)), and such fluorescence spectrum is very similar to that of compound B in aggregates in THF with f_w = 90% (Figure 2(a)), presumably resulting from the formation of same packing modes.

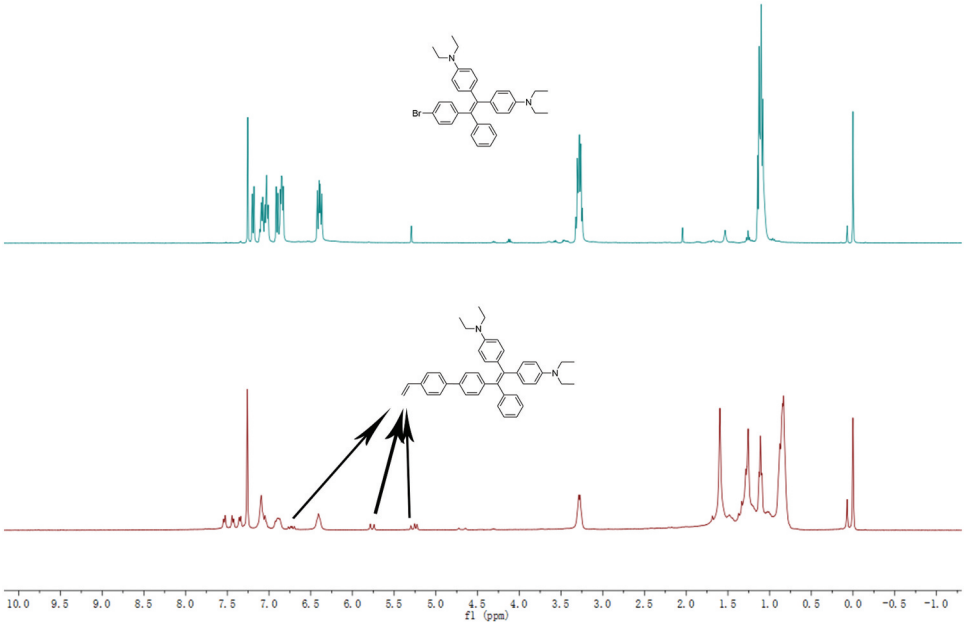


Figure 1. ^{1}H NMR of compound A (up) & compound B (bottom) recorded in $CDCl_3$.

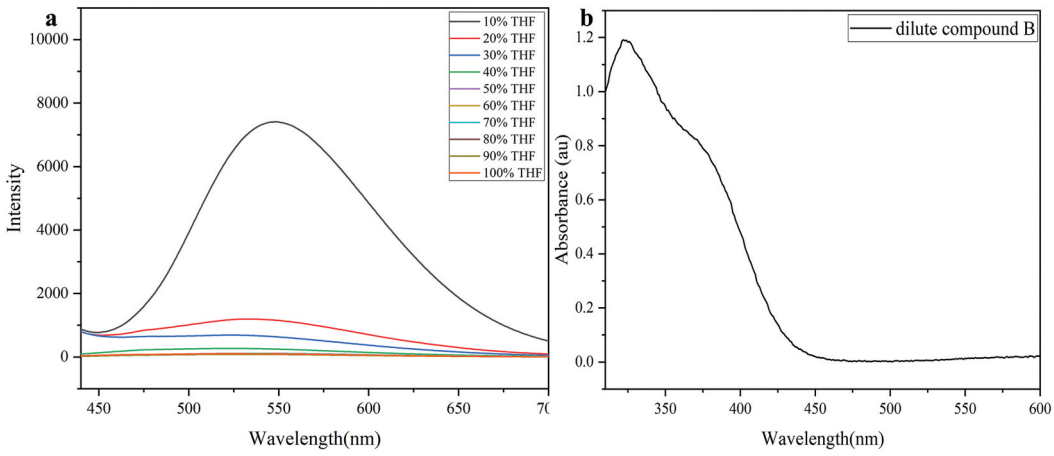


Figure 2. (a) PL spectra of compound B excited at 340 nm in water/THF mixtures with different fractions. (b) UV-vis spectra of compound B in dilute THF solution. Concentration: 10 μM.

Table 1. The size and CV of microspheres with different contents of compound B.

AIE monomer loading/mg	Size/μi	CV
0	3.00	2.8%
5	2.66	5.5%
10	2.62	6.0%
20	2.78	4.0%

3.2. Synthesis and morphological characterization of functional fluorescence-encoded polystyrene beads

The functionalized polystyrene microspheres were synthesized by two-stage dispersion polymerization using AIEgens (compound B) and styrene as monomers in ethanol in the presence of stabilizer. In order to prepare monodisperse microspheres, the compound B was added in the second stage of dispersion polymerization. The fluorescence intensity of the beads could be precisely controlled by adjusting the amount of AIEgens added. Different quantities of compound B (0–20 mg, Table 1) were used in the polymerization, respectively. The size and CV of microsphere size were summarized in Table 1 and the SEM pictures were shown in Figure 3. As shown in Figure 3, all the microspheres were spherical with smooth surface and diameters of about 3 μm, indicating no influence of morphology caused by the addition of AIE monomer. The CV was 2.8%, 5.5%, 6.0%, 4%, respectively. The low CV microshpere was propitious to biomedical application.

3.3. Fluorescent properties of AIE microspheres

The fluorescence spectra of these AIE microspheres were shown in Figure 4. As shown in Figure 4(a), the maximum of emission of AIE microspheres was about 525 nm in agreement with AIE monomer. The intensities of the AIE microspheres at 525 nm were increased with the addition of AIE monomer from 5 mg to 20 mg during preparation, indicating that the AIE monomer was copolymerized with styrene. Furthermore, the excitation spectra of AIE microspheres were different with that of the AIE monomer due to the presence of styrene unites copolymerized in the AIE microspheres

Confocal fluorescence microscopy is often used to examine the fluorescence of microspheres. Herein, the confocal fluorescence microscopic graphs of the beads were shown in Figure 5, which were obtained on the FITC channel using 400 nm excitation light. Since the AIE monomer was copolymerized with styrene, the fluorescence of the AIE microspheres was uniform and no fluorescent ring was observed, which was indicated that the AIE dye was well-distributed in the microspheres. Meanwhile, with the increase of AIE content in copolymerization, the fluorescence became brighter, which was coincident with the fluorescent spectra.

3.4. Flow cytometry detection

To detect the fluorescence dispersity of the fluorescent beads, the fluorescent beads were characterized by flow cytometer. Figure 6 showed the relationship between the AIEgens content and the fluorescence intensity of the fluorescent beads. Compared Figure 6 (a) and (b), fluorescent intensity increased dramatically when 5 mg AIE monomer (about 4 out of ten thousand to styrene) was used in the polymerization. The fluorescent intensity of the beads increased concurrently with the increasing amounts of AIEgens addition. As shown in Figure 6(e), four kinds of microspheres with different AIE content (0, 5, 10, 20 mg) were mixed together and detected on the

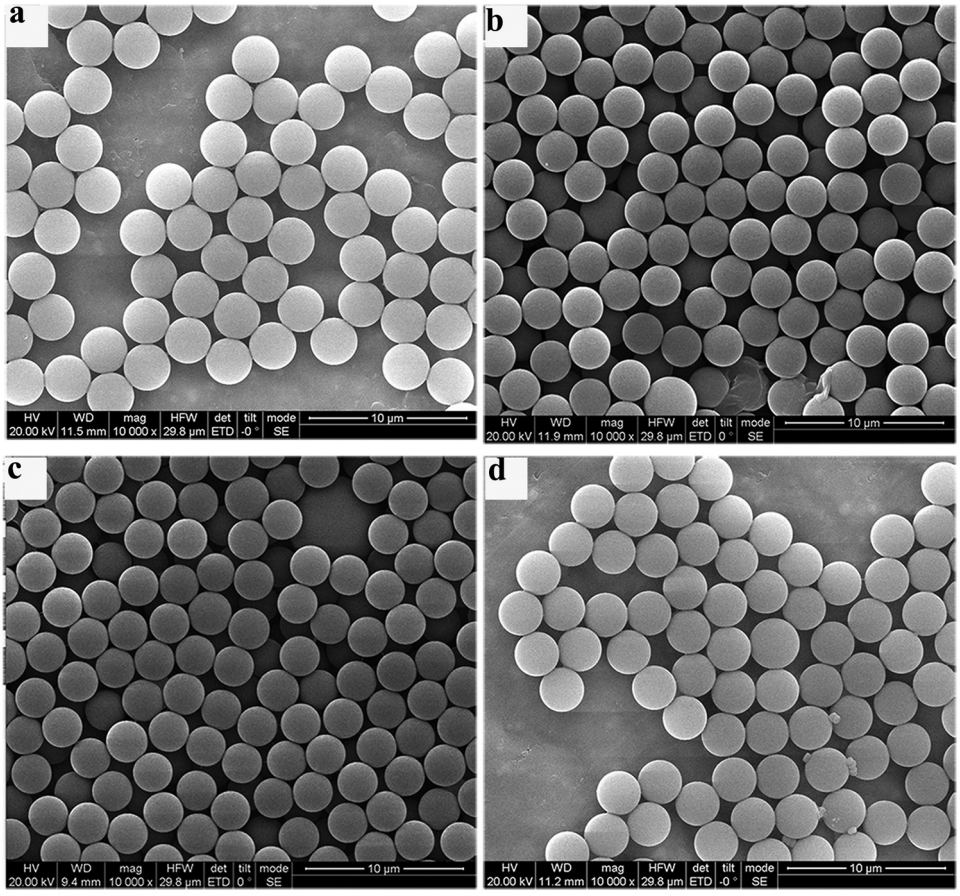


Figure 3. SEM pictures of polystyrene microspheres with different content of compoud B, 0 mg (a), 5 mg (b), 10 mg (c), 20 mg (d).

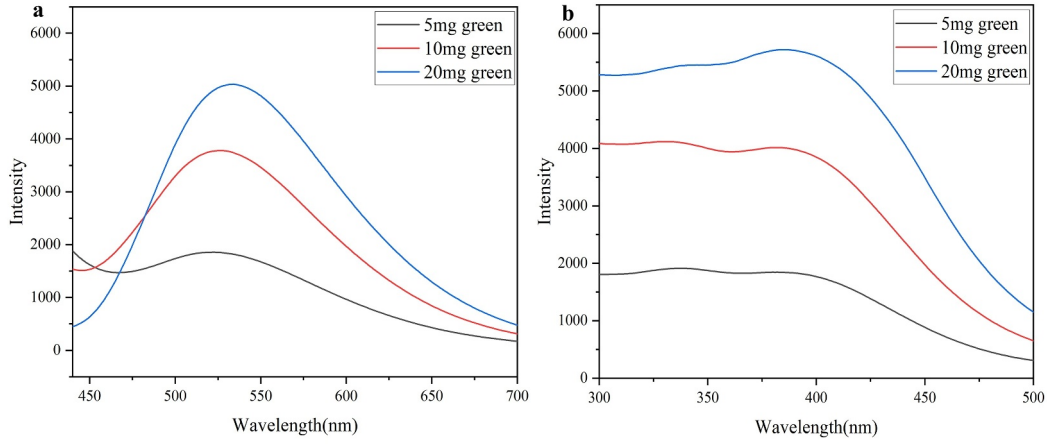


Figure 4. (a) emission spectrum of green AIEgens-microspheres, (b) excitation spectrum of green AIEgens-microspheres.

KO525 channel of the flow cytometer. It was obvious that three kinds of fluorescent beads encoded with different AIE content could be distinguished completely without obvious overlap. This result demonstrated that we could obtain fluorescence-encoded beads through the precise control of the AIE content during copolymerization.

3.5. The fluorescent stability of AIE microspheres

The fluorescent stability of AIE microspheres was estimated in water, ethanol and DMSO. In order to compare with AIE microspheres prepared by copolymerization, we prepared another kind of AIE microspheres by traditional swelling method, by which the AIE monomer (compound B) was

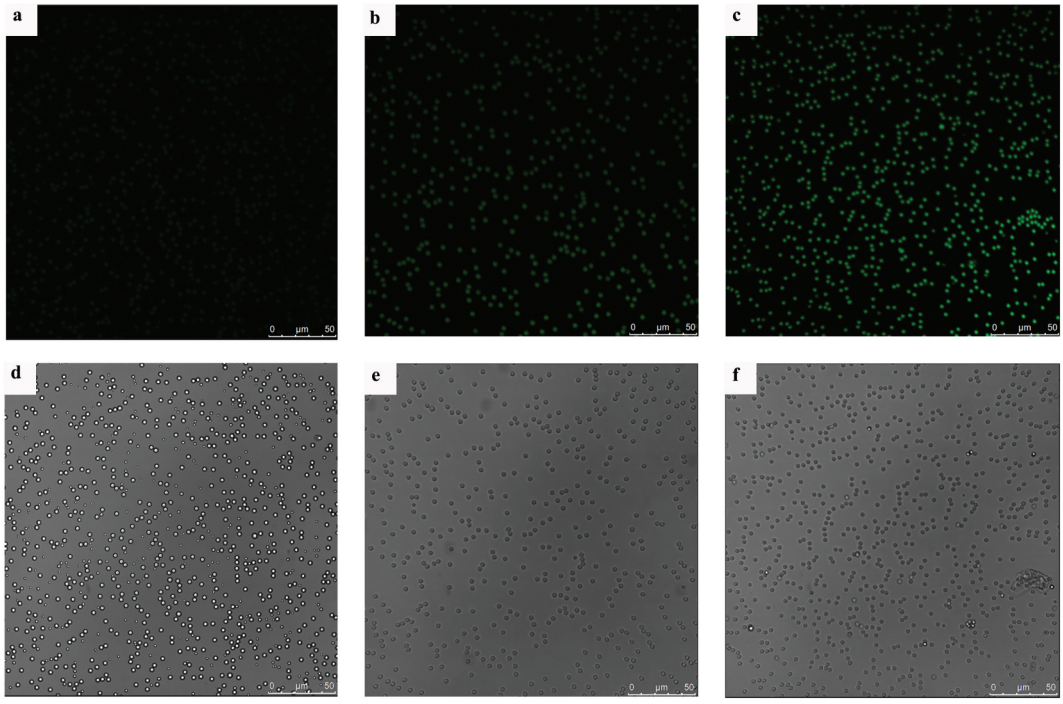


Figure 5. Confocal micrograph of fluorescent polystyrene microspheres with (a,e) 5 mg,(b,d) 10 mg, (c,f) 20 mg compoud B at same exposure time.

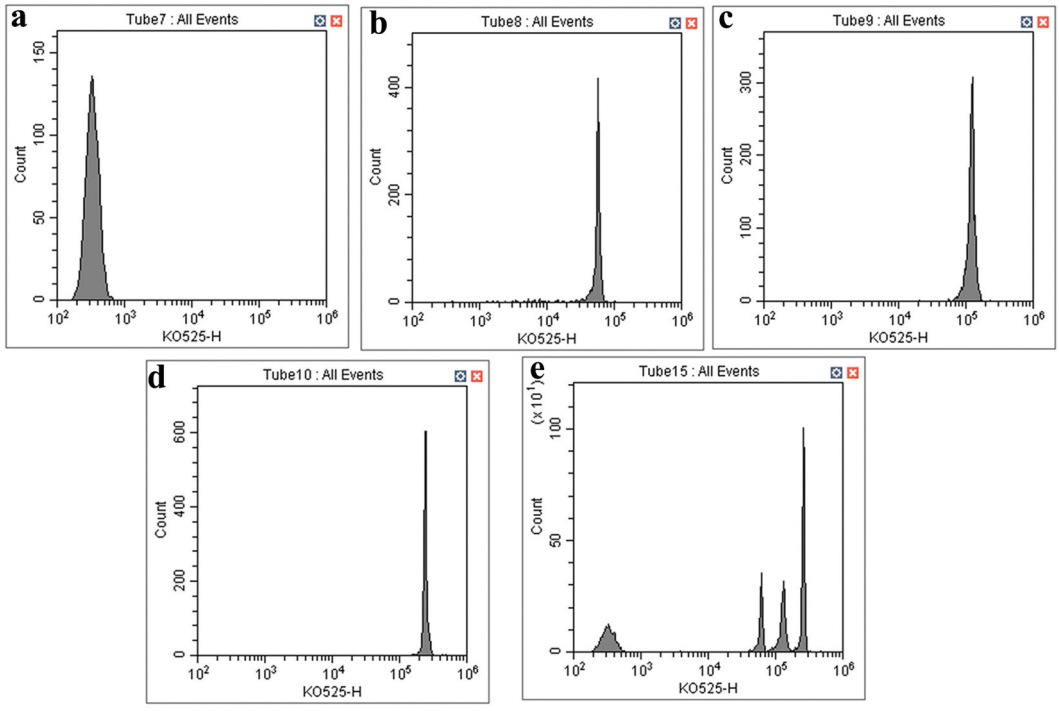


Figure 6. Fluorescence flow cytometry image of green AIEgens-microspheres with (a) blank, (b) 5 mg, (c) 10 mg, (d) 20 mg compoud B & (e) mix of all above, the different fluorescence intensities of the microspheres can be easily decoded on the KO525 channel of the flow cytometer.

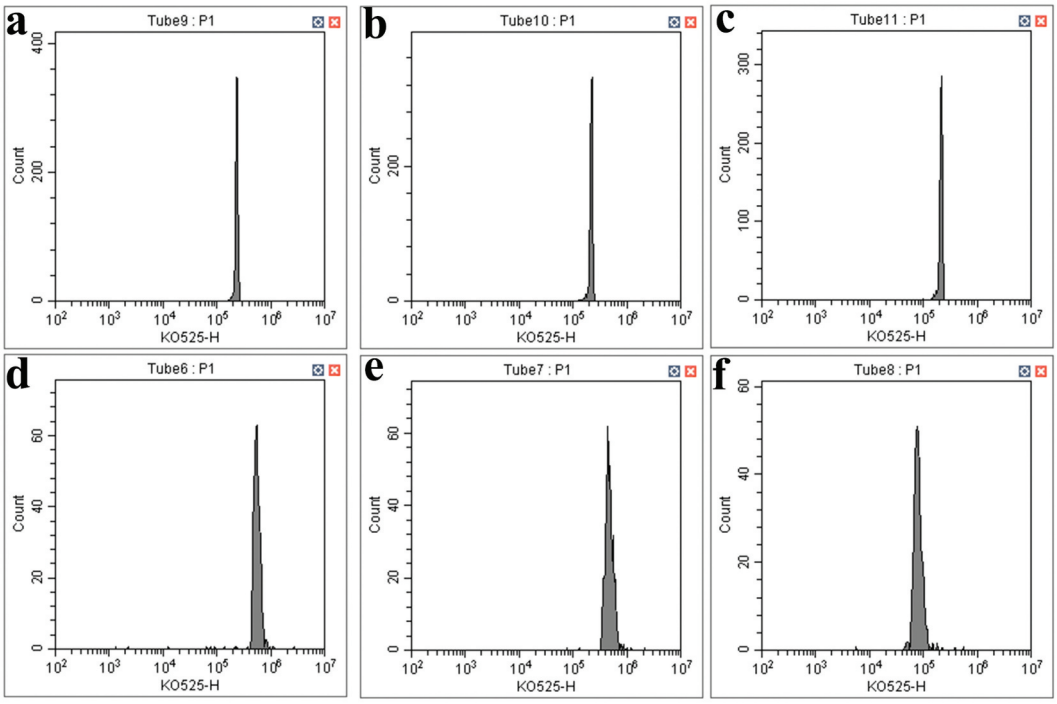


Figure 7. Fluorescence flow cytometry image of copolymerized green AIEgens-microspheres stored in (a) water, (b) ethanol, (c) DMSO and swelled green AIEgens-microspheres stored in(d) water, (e) ethanol, (f) DMSO for two weeks at room temperature, the different fluorescence intensities of the microspheres can be easily distinguished on KO525 channel of the flow cytometer.

swelled into blank microspheres. The AIE microspheres by copolymerization and swelling were placed at room temperature for two weeks in water, ethanol and DMSO respectively. The flow cytometry results were shown in Figure 7, we can see that the fluorescence of AIE microspheres prepared by copolymerization was almost consistent in water, ethanol and DMSO, and there was almost no fluorescence leakage occurred. The AIE dye in the copolymerized microspheres was stable because AIE monomers were copolymerized with styrene by strong covalent bond in the microspheres. However, the fluorescence of swelling AIE microsphere in DMSO decreased from 5×10^5 to 1×10^5 (Figure 7(f)). The decrease was probably due to the fluorescence leakage caused by the swelling of AIE microsphere in DMSO, which may be a disadvantage in further application. Furthermore, the peaks in KO525 channel of AIE swelling microspheres were wider in all these three solvents compared with that of AIE microspheres prepared by copolymerization, which indicated that the CV of fluorescence was higher. Overall, the AIE microspheres prepared by copolymerization had higher stability and lower fluorescence CV.

4. Conclusions

In the present work, a new kind of green AIE monomer was synthesized using Suzuki reaction, based on which a transition metal-free, nonhazardous, nontoxic and atom-economic method to prepare AIE-active microspheres was developed with two-stage dispersion polymerization. SEM characterization showed that the content of AIE monomer did not result in remarkable changes in either size or shape of the fluorescent beads. The particle size of the synthesized microspheres is approximately 3 μm and CV was around 5%. We obtained three fluorescence-encoded beads through the precise control of the AIEgens content during the copolymerization process. These three kinds of fluorescent beads encoded with different AIEgens content can be distinguished completely without obvious overlap using fluorescence flow cytometry. They also can be observed equidistributed in confocal microscopy, indicating the AIE dye was uniformly distributed in the microspheres and no fluorescence leakage occurred. The present work provided a general strategy to prepare AIE-based fluorescent materials with good monodispersity and fluorescence-encoded properties for biomedical imaging and other applications like fluorescent devices and organic field-effect transistors.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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