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Preparation of Fluorescence Tunable Polymer Nanoparticles by One-step Mini-emulsion

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Fluorescence tunable polymer nanoparticles were prepared by incorporating two hydrophobic fluorescent dyes (9, 10-diphenylanthracene: DPA and nitrobenzoxadiazolyl: NBD) into polymethylmethacrylate (PMMA) nanoparticles via one-step mini-emulsion polymerization method. The prepared fluorescent nanoparticles exhibit the spectral properties of both DPA and NBD dye, indicating that the two fluorophores have been incorporated into the nanoparticles. The nanoparticles greatly enhance the fluorescence emission of the two hydrophobic dyes in aqueous media probably by providing good protection of the dye molecules in the polymer nanoparticles matrix. Moreover, by varying the doping ratio of the two hydrophobic dyes, the polymer nanoparticles exhibit tunable and distinguishable emission characteristics under a single wavelength excitation via occuring fluorescence resonance energy transfer (FRET).

Keywords: Fluorescence tunable, polymer nanoparticles, FRET, mini-emulsion

1 Introduction

Nowadays, multiplexed signaling and bioanalysis play an important role in many applications, such as genetic detection, high-throughput screening in drug discovery, and clinical diagnosis (1–5). The multiplexed bioassays often need plenty of fluorescent labels, which have single wavelength excitation and distinguishable emission signal. However, the amounts of the conventional organic dyes that meet this requirement are limited. In addition, the emission signal of organic dye molecules is weak, and the dye molecules are vulnerable to irreversible photodestruction (1). On the other hand, Quantum dots (QDs) have gained great interest in this field due to their broad excitation spectra and tunable emission wavelengths by a single wavelength excitation. However, QDs "blinking" characteristic as well as cytotoxicity is a limiting factor *in vivo* application (6,7).

To address these issues, fluorescence tunable nanoparticles have been developed to serve as alternative substrate for multiplexed bioassays by incorporating two or more energy transfer fluorescent dyes into the single nanoparticle (8–13). In comparison with conventional organic dyes, fluorescence tunable nanoparticles have exhibited several advantages. For instance, they not only exhibit high brightness, improved photostability and biocompatibility, but also feature versatility in design and synthesis, and high sensitivity might be achieved since a large number of dye molecules can be incorporated into a single particle (8). Moreover, by varying the doping ratio of dyes, it is possible for nanoparticles to generate versatile colors with tunable emission signatures by FRET under a single wavelength excitation (8,10). Then a large number of encoding nanoparticles population can be generated from a limited number of individual fluorophores. For example, Tan et al. (8) used FRET-mediated silica nanoparticles which exhibit multiple colors under single wavelength excitation, and utilized it in multiplexed bacteria monitoring (9). Larpent et al. (13) synthesized and researched dual fluorophore-doped polystyrene nanoparticles with emission features which can be tuned by varying the doping ratio via FRET, and used it as cascade FRET-mediated ratiometric sensor for cupric ions in water. Zhang et al. (10) synthesized multicolor upconversion fluorescent nanoparticles by doping organic dyes or QDs into the silica shell of $NaYF_4$:Yb, Er/Tm nanoparticles, and the different upconversion fluorescence was generated through FRET from the $NaYF_4$ core to the organic dyes or QDs.

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The above mentioned fluorescence tunable nanoparticles were generally obtained by incorporating multiple dyes into nanoparticles via covalent linkage, or physical doping during the synthesis process or by modification of nanoparticles. However, the conventional approaches usually involve relatively complicated procedures (10), and sometimes it is difficult to control the quantity and the ratio of the different dyes in the nanoparticle system (11). Recently, mini-emulsion technology provides a promising method to address some of the problems. Mini-emulsions were aqueous dispersion of relatively stable and small (50∼ 500 nm) oil (oil-soluble monomer) droplets prepared by using high-force dispersion devices (14–16). Each of the droplets can be regarded as an individual batch reactor, a variety of substances (including reactable monomers or various non-reactable functional species) can be introduced into individual mini-emulsion, and then be subject to polymerization to obtain polymer particles. So far, mini-emulsion polymerization has been widely applied for encapsulation of various functional groups, especially hydrophobic dyes, into polymer particles (17–23). For example, Landfester et al. (17) incorporated the magnetic nanoparticle and fluorescent dye into polymeric nano-beads via mini-emulsion polymerization and obtained fluorescent and magnetic dual report nanoparticles usable in the biomedical field. Tamai and coworkers (18) prepared dual-dye-doped (pyrene and alkynylpyrenes) fluorescent nanoparticles by mini-emulsion polymerization, and the nanoparticle system show high fluorescence quantum yield. Kawaguchi et al. (19) prepared high-performance fluorescent polymer particles with Eu beta-diketonates complex as a fluorophore by mini-emulsion polymerization technique. We (20) also synthesized photoswitchable fluorescent nanoparticles by simultaneously incorporated spiropyran molecules and NBD dye into the polymer nanoparticles via mini-emulsion polymerization method, and the amount and ratio of the two chromophores in the nanoparticles can be well controlled.

In this article, we successfully prepared a series of fluorescence tunable polymer nanoparticles included two hydrophobic fluorescent dyes by a one-step mini-emulsion polymerization. The selected dyes are two well known and widely studied hydrophobic dyes with high quantum yield: 9,10-diphenylanthracene (DPA) and nitrobenzoxadiazolyl (NBD) (24–26). This facile method can lead to the fluorescence tunable nanoparticles with smaller size (ca. 80 nm), higher dye load, as well as controllable amount and ratio of the two dyes. Moreover, by varying the doping ratio of two dyes, the FRET-mediated fluorescent nanoparticles exhibit multiplex colors as well as fluorescence emission signals under a single wavelength excitation.

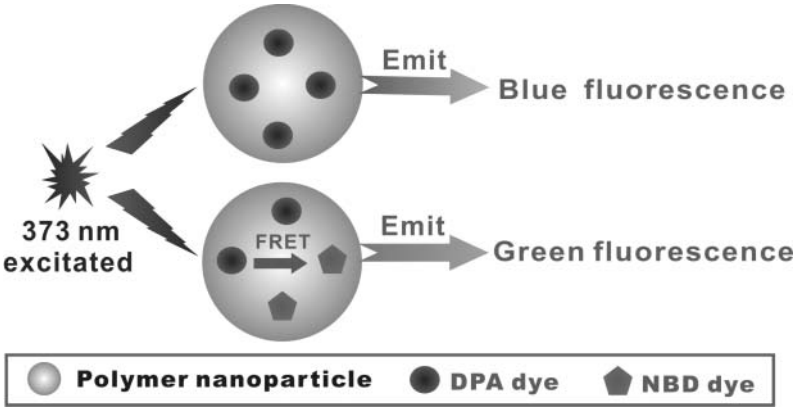
2 Experiments

2.1 Materials

The surfactant sodium dodecyl sulfate (SDS, 99%, Aldrich), n-hexadecane (HD, 99%, Aldrich), potassium persulfate (KPS, 99.99%, Aldrich), 9,10-diphenylanthracene (DPA, Aladdin) were used as received. The methyl methacrylate (MMA, Aldrich) was purified by distillation under vacuum to remove inhibitors. Dichloromethane (A.R.) was washed with sulfuric acid and then distilled from CaH_2. Nitrobenzoxadiazolyl (NBD) dye was synthesized following the previously reported procedure (20). The water used in this work is double-distilled water, which was further purified with a Milli-Q system. And other reagents are analytical reagents and used without further purification.

2.2 Preparation of Fluorescence Tunable Polymer Nanoparticles

A mixture containing the monomers and hydrophobes (MMA, DPA, NBD, HD) was added to water solution with



Sch. 1. A) Schematic illustration of fluorescence tunable polymer nanoparticles containing one or two fluorescent dyes (DPA) and (NBD) at a single wavelength excitation.

emulsifier (SDS) and stirred (1000 r/min) for 15 min, then the mixture was ultrasonicated for 30 min (KQ2000DE) to obtain a mini-emulsion. The mixture was cooled in an ice-bath during ultrasonification to avoid being heated. The resulting mini-emulsion was put into a 50 mL flask equipped with a condenser, which was immersed in an oil bath with a thermostat. The polymerization was started by adding aqueous solution of KPS and preceded at 60°C for 210 min. After the polymerization, the as-prepared nanoparticle dispersions were percolated and finally the nanoparticle dispersions were obtained.

2.3 Characterization

The nanoparticle diameters were determined by a Malvern Nano-ZS90 instrument and their morphology was observed with an atomic force microscope (AFM, Seiko SII 400) in the tapping mode. UV-Vis spectra was recorded on a Shimadzu UV-2501PC spectrophotometer at room temperature. Fluorescence spectra was recorded on a Shimadzu RF-5301PC fluorescence spectrophotometer at room temperature.

3 Results and Discussion

3.1 Preparation of Fluorescence Tunable Polymer Nanoparticle Dispersions

To synthesize the fluorescence tunable polymer nanoparticles, two hydrophobic fluorescent dyes (DPA, NBD) with different ratios were mixed with MMA and HD dispersed into water with surfactant under ultrasonic shear force, and then the initiator KPS was added to initiate the mini-emulsion polymerization of the monomer mixture. Finally, nanoparticles incorporated with one or two fluorescent dyes were obtained. These nanoparticles emit blue fluorescence when one dye (DPA) was incorporated into nanoparticles under excitation at 373 nm, while they emit green fluorescence when two dyes (DPA and NBD) with determined ratio were incorporated into them by means of FRET under exciting at 373 nm, as illustrated in Scheme 1.

Through typical mini-emulsion polymerization, nanosized to submicron-sized polymer particles can be obtained (14). The size of polymer particles can be controlled by varying the amount of surfactant and hydrophobe, the monomer concentration in water, as well as the ultrasonic time (14). For the fluorescent nanoparticles, their spectral properties can be affected by the particle size because of the scattering light (wavelength = λ) by a particle is maximum when the diameter of the particle is $\lambda/2$ (17). In order to eliminate the effect of scattering light, all the fluorescent nanoparticles are almost uniform in diameter by optimizing some experimental parameters, the average diameter of the prepared nanoparticles is around 77 nm, as determined by dynamic light scattering (DLS) (Table 1). Moreover, it can be seen from Table 1 that the presence of dye had a little influence on the particle size.

Figure 1**A** shows an AFM image for a typical nanoparticle sample (NP-F), as can be seen from the image that the polymer nanoparticles of the sample were discreet, regular spherical with the diameters of 65–80 nm, and its stable dispersion with an average diameter of 77.1 nm is determined by DLS (Fig. 1(B)). The results of the AFM experiment are in good agreement with DLS data, indicating mini-emulsion is a promising method to prepare uniform polymer nanoparticles with defined size.

Table 1. List of nanoparticle samples incorporated two fluorescent dyes in this work

	DPA [mg]		*NBD [mg]*		C_{DPA}/C_{NBD}^{d}			
Sample[a]	*Feed*	*Determined*[b]	*Feed*	*Determined*[c]	*Feed*	*Determined*	*Dm*[e] *[nm]*	*PDI*[e]
NP-A	4.0	2.9	0	0	1:0	1:0	76.4	0.076
NP-B	4.0	3.0	0.7	0.5	1:0.2	1:0.18	78.9	0.072
NP-C	4.0	3.1	1.4	1.1	1:0.4	1:0.40	75.4	0.112
NP-D	4.0	3.2	2.1	1.7	1:0.6	1:0.60	80.8	0.105
NP-E	4.0	3.0	2.8	2.3	1:0.8	1:0.86	80.3	0.115
NP-F	4.0	3.1	3.5	3.0	1:1	1:1.09	77.1	0.116
NP-G	0	0	3.5	2.9	0:1	/	79.8	0.089

[a]The MMA/HD/SDS/KPS feed is 2.5/0.15/0.05/0.085g, respectively.
[b]Calculated by using the absorbance of DPA at 375 nm in nanoparticle dispersion (eliminate the effect of scattering light) and the molar extinction coefficient of DPA in dichloromethane, ($\varepsilon = 15500\ mol^{-1}.L.cm^{-1}$).
[c]Calculated by using the absorbance of NBD at 459 nm in nanoparticle dispersion (eliminate the effect of scattering light) and the molar extinction coefficient of NBD in dichloromethane ($\varepsilon = 22000\ mol^{-1}.L.cm^{-1}$).
[d]Mole concentration ratio of DPA/NBD (assume DPA is 1).
[e]Average nanoparticle diameter, determined from DLS data.

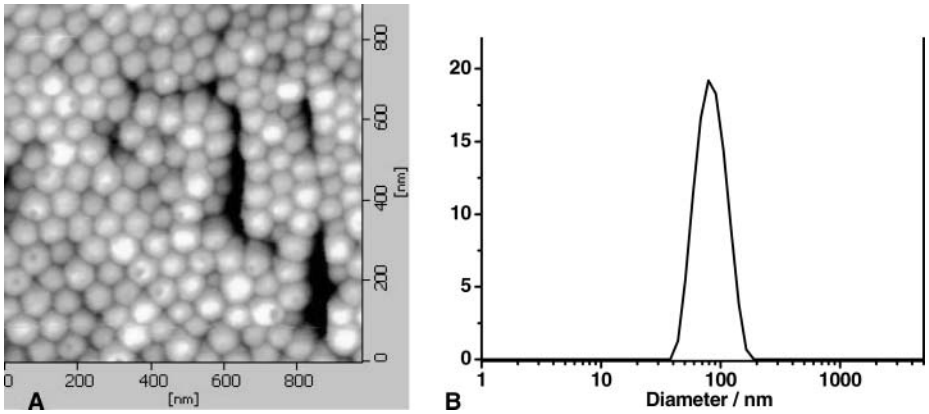


Fig. 1. A) AFM image of two-dye-doped nanoparticles (sample NP-F), **B**) Size distribution for the nanoparticle sample NP-F determined by DLS.

3.2 Spectroscopic Properties of Fluorescence Tunable Nanoparticles

To detect whether two hydrophobic fluorescent dyes could be co-encapsulated into nanoparticles by a mini-emulsion polymerization method, we used two hydrophobic fluorescent dyes (DPA and NBD) as a model in this study. It is well known that the DPA is served as typical hydrophobic fluorescent dye which is almost unsolvable in water because of its symmetrical non-polar structure. As for the NBD dye, with a nitrobenzoxadiazolyl group and an 8-carbon alkyl tail, also exhibits very low solubility in pure water due to its hydrophobic nature. So, as shown in Figure 2, there is no reliable absorption spectrum produced from their saturated water solution. Figure 2 also shows the absorption spectra of DPA and NBD in dichloromethane solution and in nanoparticle dispersions. The nanoparticles, which incorporate two hydrophobic fluorescent dyes by mini-emulsion polymerization method, can drastically increase the solubility of the two dyes in the aqueous dispersion and enhance their UV-Vis absorption spectrum in aqueous media. Moreover, with the incorporation of two dyes into the nanoparticles, the nanoparticles containing dyes showed three prominent absorption peaks at 375 nm, 393 nm, and 459 nm, that are close to that for DPA and NBD in hydrophobic solution like dichloromethane, but display obviously blue-shift as compared to that for DPA and NBD in water, implying that the dye molecules reside in a more hydrophobic environment.

Figure 3 indicates the fluorescence spectra of DPA and NBD in pure water, in dichloromethane solution and in nanoparticle dispersions, respectively. Due to the very low solubility of two dyes in pure water, their saturated

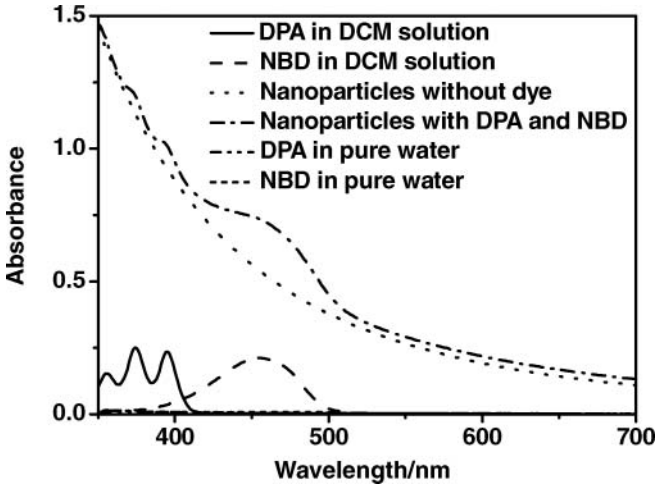


Fig. 2. Absorption spectra for DPA dye and NBD dye in pure water, in dichloromethane solution and in nanoparticle dispersions, respectively, the spectrogram show that the decrease in the absorbance curve from short to long wavelength is due to the light scattering effect of nanoparticles in water.

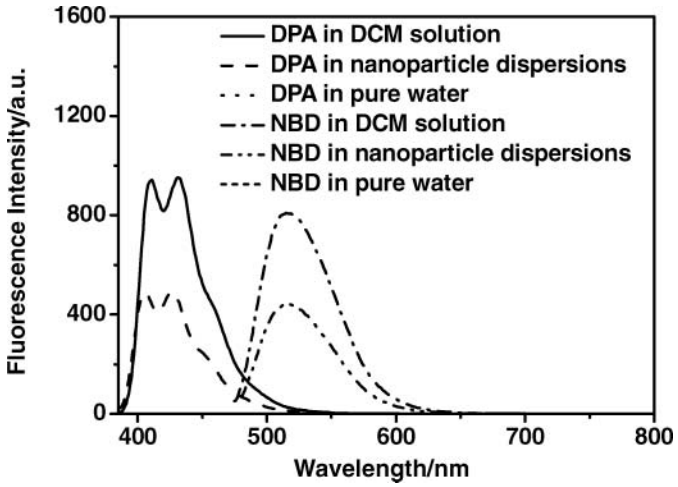


Fig. 3. Fluorescence emission spectra of the DPA dye and NBD dye in pure water, in dichloromethane solution and in nanoparticle dispersions respectively. The fluorescence signals of DPA and NBD in pure water were neglectable.

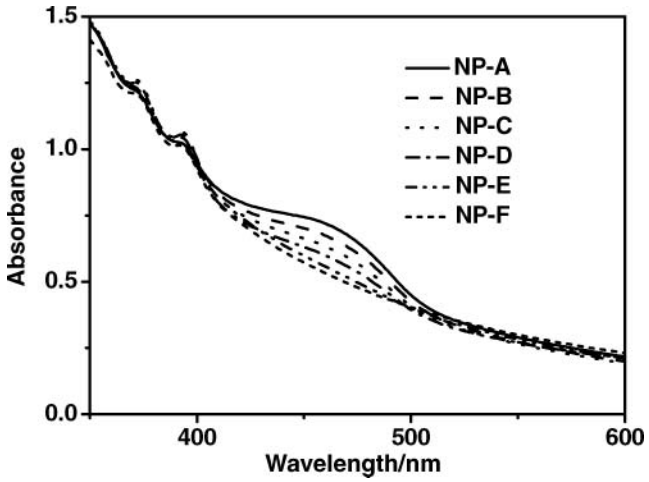


Fig. 4. Absorption spectra for six nanoparticle samples with the same DPA feed and different NBD feeds (from sample NP-A to NP-F, the NBD feed increasing).

aqueous solution exhibit unobvious fluorescence emission intensity. However, with the incorporation of two dyes into the nanoparticles respectively, the nanoparticles show intense fluorescence, and the fluorescence property is similar to the dye in dichloromethane solution. The results suggest that the dye molecules reside in the more hydrophobic environment like polymer nanparticle matrix.

The amount (content) of fluorophores incorporated into the nanoparticles can be deduced by using the absorption values assuming that the molar extinction coefficient of fluorophore in nanoparticles is the same as that in some organic solutions (18, 24). In the present study, the amounts of the two fluorescent dyes incorporated within the nanoparticles are also deduced by using the extinction coefficient of NBD or DPA dye in dichloromethane. As shown in Figures 2 and 4, the nanoparticle dispersions exhibit a light scattering effect, so the actual absorbance values for DPA (at 375 nm) and NBD (at 459 nm) can be obtained by eliminating the light scattering effect. Table 1 listed the determined amount of DPA and NBD in nanoparticle samples via the absorbance values. The amounts of DPA and NBD calculated through absorption measurement for the nanoparticle samples are about 71∼86% of the feed amount, and we think the difference may arise from the systematic error of this determination method or the leakage of dyes during polymerization; On the other hand, as shown in Figure 4, with the gradually increasing of NBD feed, the absorbance value is also added by a certain proportion, and the determined concentration ratio of DPA to NBD dye is close to the feed ratio (Table 1). These results indicate that the mini-emulsion polymerization is suitable for encapsulation of two fluorophores (DPA and NBD), with their ratios close to pre-designed values, into polymer particles.

3.3 FRET in Polymer Nanoparticles

As shown in Figure 5, the fluorescence emission band of the DPA (350–550 nm) overlaps well with the absorption band of the NBD (350–550 nm) in nanoparticles (Fig. 5). According to Förster's theory (3), DPA and NBD are suitable as donor and acceptor, respectively. If keeping them within the effective energy transfer distance (generally, 1 nm to 10 nm), it is possible to obtain two tunable emission signatures with a single wavelength excitation by FRET from DPA to NBD.

In order to research the FRET process between DPA dye (donor) and NBD dye (acceptor) in nanoparticles, three nanoparticle samples NP-A, NP-F and NP-G (Table 1) were prepared. The fluorescence emission spectras of three samples are displayed in Figure 6. The donor and acceptor concentrations of sample NP-F (with DPA and NBD) are close to that single-dye-incorporated sample NP-A (only

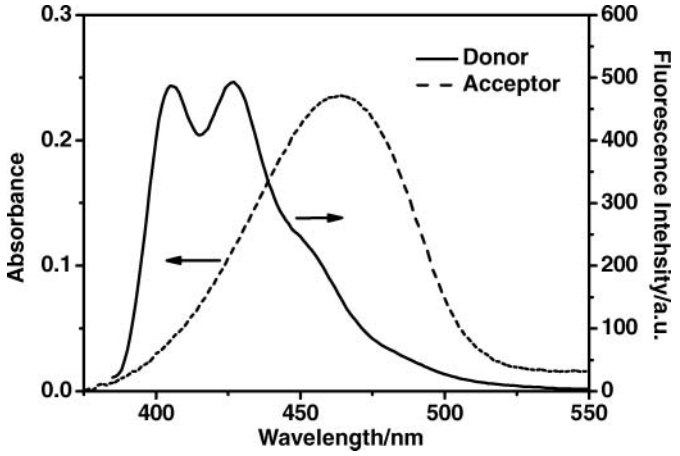


Fig. 5. Emission spectrum of DPA (solid) and absorption spectrum of NBD (dashed) in nanoparticles.

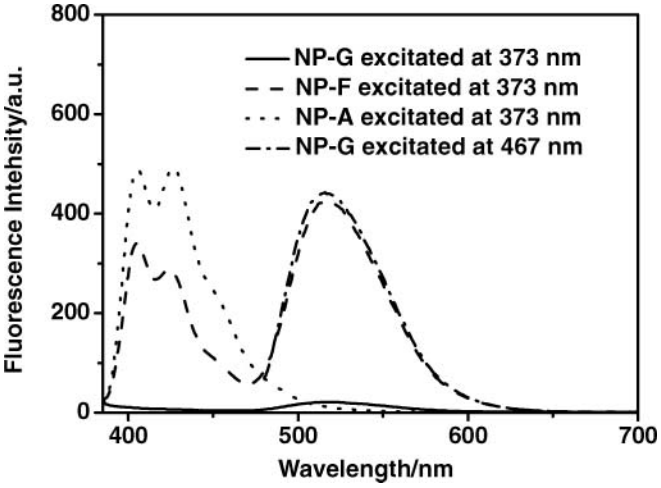


Fig. 6. Fluorescence spectra of three representative samples: Sample NP-A, Sample NP-F and Sample NP-G.

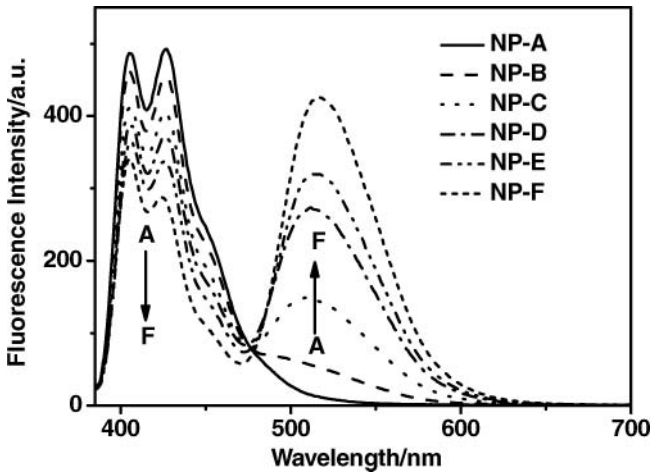


Fig. 7. Fluorescence emission spectra of six nanoparticle samples with different NBD feeds (from samples NP-A to NP-F, the NBD feed increasing).

with DPA) and sample NP-G (only with NBD). As shown in Figure 6, the sample NP-A exhibits strong fluorescence emission intensity at 405 nm and 426 nm by excitated at 373 nm. In contrast, the sample NP-G only shows low fluorescence emission intensity at 516 nm. But we found that the sample NP-G indicates strong fluorescence at 516 nm when a longer excitation wavelength of 467 nm was adopted. Generally, as for the sample NP-F, it should exhibit strong fluorescence emission of DPA and weak emission of NBD as like that of samples NP-A and NP-G when excitated at 373 nm. However, the sample NP-F actually shows decreased emission intensity of DPA (donor) and dramatically increased emission intensity of the NBD (acceptor). It demonstrates that the determinate FRET process occurs from the donor (DPA) to acceptor (NBD) in a single nanoparticle system.

3.4 Fluorescence Tunable Emission Signatures of Nanoparticle Dispersions

For multiplexed bioassays of numerous analytes, a large number of nanoparticle populations with distinguishing fluorescent characteristics are required. By using the immobile UV illumination or laser excitation, these nanoparticles should exhibit different emission signals. In this study, a series of fluorescence tunable monodisperse polymer nanoparticles were prepared in terms of FRET in nanoparticles. Figure 7 shows the fluorescence spectra of six samples (from NP-A to NP-F) by a single wavelength excitation of 373 nm. It can be seen from the fluorescence spectra that the band centering at approximately 405 nm and 426 nm are the characteristic emission of DPA dye and 516 nm is the characteristic emission of NBD dye. By variation of two dyes ratio in nanoparticles, the fluorescence emission property of the nanoparticle dispersions can be correspondingly varyed. With increasing the doping ratio of DPA and NBD from 1:0 to 1:1, the fluorescence emission intensity of donor gradually decreased and the acceptor emission intensity steadily increased, at the same time, the color of nanoaprticle dispersions under 365 nm UV light excitation also gradually changed from blue to green (Fig. 8). So, it is feasible to obtain tunable and distinguishable emission characteristics, as well as different colors through FRET by varying the two dyes ratio in nanoparticles. These nanoparticles are useful to identify specific target analytes in biological application area with their unique emission characteristics.

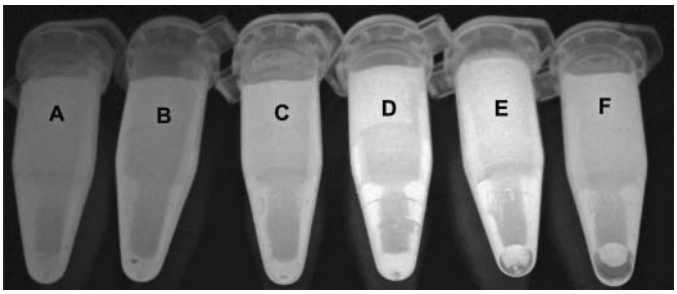


Fig. 8. Images of Samples NP-A to NP-F at the same particle concentration under 365 nm UV illumination.

4 Conclusions

In conclusion, the fluorescence tunable nanoparticles containing the DPA and NBD dyes were successfully prepared by a facile one-step mini-emulsion polymerization. The fluorophores' amount and their ratio within individual nanoparticles can be readily controlled through this strategy. By varying the doping ratio of two dyes, the FRET-mediated polymer nanoparticles exhibit tunable fluorescence emission signatures under a single wavelength excitation. These fluorescence tunable nanoparticles may find applications in biological areas such as multiplexed biological analysis, imaging and detection.

Acknowledgments

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