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Preparation of Cross-Linked, Multilayer-Coated Fluorescent Microspheres with Functional Groups on the Surface for Bioconjugation

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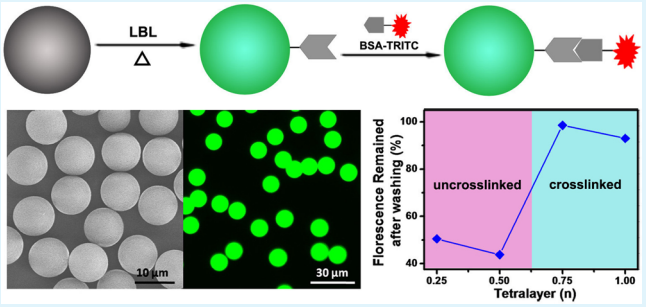
Supporting Information

ABSTRACT: This study is to develop a method for preparing fluorescent microspheres with steady and strong fluorescence as well as the surface functionality for bioconjugation. Layer-by-layer technique was employed to introduce poly(phenylenevinylene) (PPV) precursor, diazoresin (DAR), and polyanion, including poly(acrylic acid) (PAA) and poly(sodium-p-styrenesulfonate) (PSS), onto the substrate polystyrene-divinylbenzene microspheres with sulfonic groups on the surface (SPSDVB). The conversion of PPV precursor into fluorescent PPV as well as the cross-linking reaction between DAR and polyanion, were accomplished simultaneously in the following thermal treatment. After optimizing the DAR concentration, the selection of polyelectrolytes and the coating sequence, the cross-linked multilayer coated PPV microspheres, SPSDVB-(PPV/PSS/DAR/PAA) spheres, were prepared. These spheres were found to have uniform size with a clear core−shell structure and display even and strong fluorescence, based on the characterization by flow cytometry, microscopy, and photophysics. They were found to be stable and highly resistant to common solvents and even "dissociation agent", as well as possess good thermal stability and photostability. The feasibility of conjugating biomolecules on the surface of spheres was also demonstrated.



KEYWORDS: *fluorescent microsphere, layer-by-layer, cross-linking, bioconjugation, flow cytometry*

1. INTRODUCTION

Together with the enjoyment of the facility and convenience brought by the rapid development of modern society, human beings are also suffering many accompanying healthcare issues and environmental problems. Flow cytometry is a well-established, effective, and efficient method for disease diagnosis and immunoassay.[1−5] Fluorescent microspheres are the key carriers for interacting with the targeted biomolecules as well as giving out the detectable signals for clinical analysis using flow cytometry.[6−12] However, the commercial fluorescent microspheres are not sufficient for meeting the huge demand, because of the limited production. There are some strict requirements for the properties they should have, such as monodispersity in micrometer size, steady and strong fluorescence with good uniformity, as well as the functionality for bioconjugation.[13−15]

Our group has been devoted to developing various methods for fabricating microspheres with desired properties to make flow cytometry serve for more people. Different from most reports using organic dyes or quantum dots as the emission species,[16−18] we selected conjugated polymers as the fluorophores. Conjugated polymers usually have broad absorption and stable emission, tunable fluorescence and good processability.[19−27] Previously, we have successfully prepared poly(phenylenevinylene) (PPV) and polydiacetylene (PDA) coated fluorescent microspheres through electrostatic interaction, physical adsorption or other weak interactions.[28−30] In these systems, we circumvented the solubility problem of PPV or PDA by using water-soluble polymer PPV precursor (pre-PPV) or water dispersible PDA micelle to realize the processability. Comparing PDA system with PPV system, PDA spheres had the carboxylic groups on the surface for interacting with biomolecules, whereas PPV spheres had relatively stronger and steadier emission. The layer-by-layer (LBL) technique is a well-developed method to introduce different layers onto a solid substrate via weak interactions (e.g., electrostatic interaction).[31−33] LBL should be a facile way to introduce different fluorophores or functionalities onto substrate spheres. However, the polyelectrolyte deposited on the spheres may be more or less washed off by solvents used during the application

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process. Therefore, the stability in both emission and functionality of the spheres can be greatly improved if the assembled layers are covalently bonded instead of electrostatically attracted.

Here we present our strategy for obtaining stable fluorescent microspheres with functional groups on the surface using LBL technique followed by thermal treatment. The schematic diagram for preparing multilayer coated spheres is shown in Figure 1. One layer of the positive charged pre-PPV, one layer

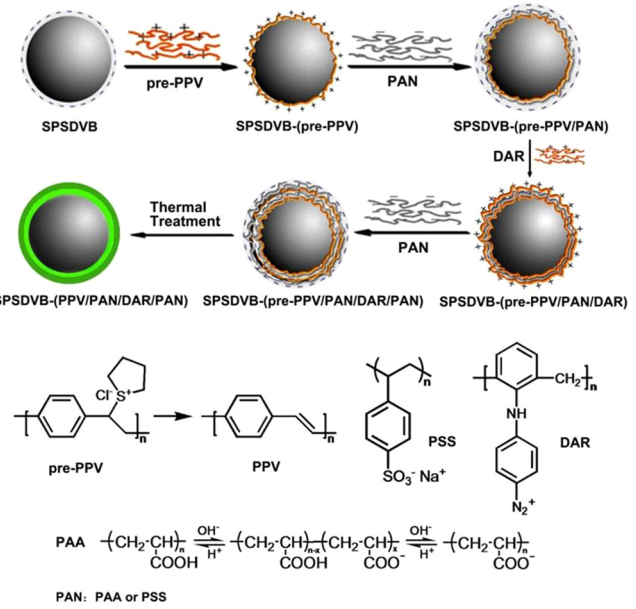

Figure 1. Schematic diagram for preparing tetralayer coated microspheres followed by thermal treatment to give the cross-linked shell coated fluorescent PPV microspheres (top) and the chemical structures of materials used in this process (bottom). PAN refers to polyanion, including PAA and PSS. PAA has different forms under different pH environments.

of polyanion (PAN), one layer of positive charged diazoresin (DAR) and another layer of PAN are consecutively deposited onto the substrate polystyrene-divinylbenzene microspheres (SPSDVB) with negative charges on the surface. Afterward, the thermal treatment is carried out to convert pre-PPV into fluorescent PPV. Meanwhile the cross-linking reaction between DAR and PAN takes place (Scheme S1 in the Supporting Information). Some advantages are anticipated for the preparative strategy. First, the cross-linking reaction between PAN and DAR, and the conversion from pre-PPV to PPV, can be accomplished simultaneously by heating the spheres, which makes the preparative process very efficient. Second, the cross-linking produces a stable protective shell to reduce possible desorption and photobleaching of the inner PPV layer. Third, the emission properties and the surface functionality can be optimized simply by varying the combination of different layers. In addition, similar to our previous systems,[28−30] the size and monodispersity of final fluorescent microspheres can be easily realized if the LBL process is well-controlled by using the commercial monodispersed spheres as the core. All these advantages make this strategy very promising for preparing fluorescent spheres to be readily used in flow cytometry based biorelated applications.

In this study, the fluorescent microspheres with surface functionality for bioconjugation were prepared according to above strategy. To obtain information useful for practical information, we used flow cytometry as the major characterization method. The emission intensity of the resulting fluorescent microspheres provided by flow cytometry was based on a statistical analysis of more than 10 000 microspheres for each measurement. Laser scanning confocal microscopy, regular emission spectra and SEM were also used to obtain more information about fluorescence and the morphology. Studies about solvent resistance, the interaction between the spheres and biomolecules, as well as thermal stability and the photostability were also carried out. All these studies provided a comprehensive evaluation for the possibility for using these spheres in practical applications.

2. RESULTS AND DISCUSSION

2.1. Optimizations in the Preparation. The pre-PPV solution was prepared according to a routine procedure in our lab.[28] The adsorption of the first layer onto the substrate SPSDVB spheres was carried out with the presence of 0.2 M NaCl in pre-PPV solution. The widely used polyanions, PAA and PSS, were selected as the polyanions based on the consideration that both of them can form cross-linking structure with DAR (see Scheme S1 in the Supporting Information for the cross-linking reactions) as well as they have functional groups for interaction with analytes. The acidic PAA solution (15% in weight percentage, pH ≈ 3) and the neutral PSS (7.5% in weight percentage) solution were used as the polyanion dipping solutions. The optimization of DAR concentration ([DAR]) used in this system was carried out. Different from the literatures using DAR to prepare cross-linked multilayer coated films,[34−37] here we mainly focused on how [DAR] influenced the final emission properties of the spheres. A series of SPSDVB-(PPV/PAA/DAR/PAA) spheres were prepared with [DAR] (in mass percentage) set at 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, and 2%, respectively, by fitting the feeding ratio among other components as pre-PPV-(mL):SPSDVB(g):PAA(mL) = 2.50:0.075:2.5. The SEM (Figure S1 in the Supporting Information) and fluorescent images (Figure S2 in the Supporting Information) of the whole series of spheres show that there is no apparent difference among them, in both morphology and emission. Photophysical studies show that all the emission spectra have similar emission profile but the emission peak red-shifted from the initial 498 to 513 nm (Figure S3 in the Supporting Information) with the increase in [DAR]. However, emission intensity measured by flow cytometry decreased greatly with the increase of [DAR] (Figure 2a). The slight red-shift in the spectra can be attributed to the extension of the π conjugation system because of the rigidification of the polymer chain during the cross-linking reaction. The absorbance peak of DAR is around 500 nm (Figure S4 in the Supporting Information), in the same range of emission maximum of the above spheres, which may account for the decrease in emission intensity with the increase in [DAR]. However, the higher [DAR], the more cross-linking takes place, which is favorable for obtaining a more stable cross-linked shell for PPV. As a compromise between the emission intensity and the stability, we decided to use [DAR] = 0.1% (in weight) solution in our study.

To compare the influence on the fluorescence intensity of spheres from PAA and PSS, we prepared two series of fluorescent spheres with cross-linked shell, SPSDVB-(PPV/PAA/DAR/PAA)$_n$ and SPSDVB-(PPV/PSS/DAR/PSS)$_n$, via consecutive adsorption of pre-PPV, PAA or PSS, DAR, and

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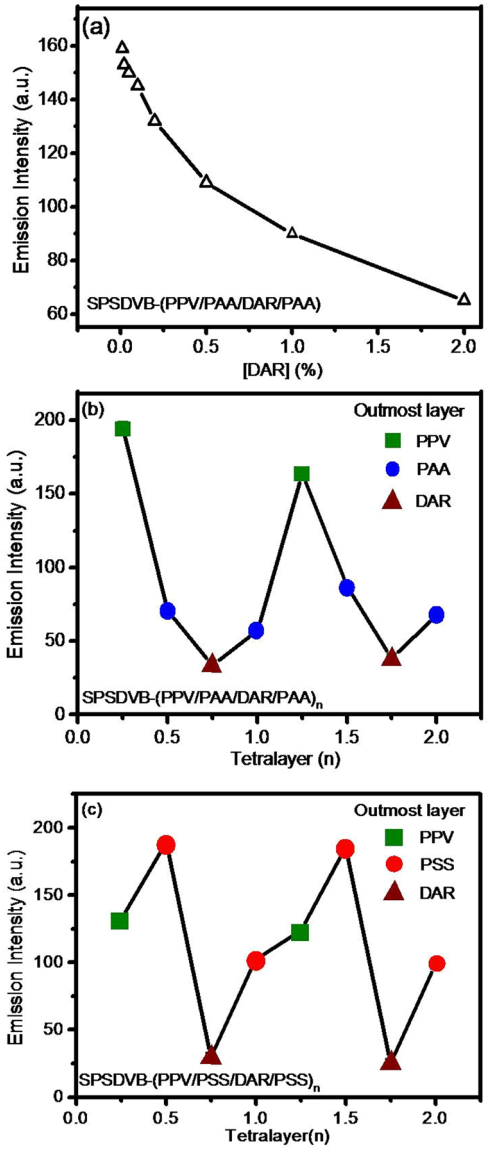

Figure 2. Fluorescence intensity from flow cytometry measurements for (a) SPSDVB-(PPV/PAA/DAR/PAA) spheres obtained using different DAR concentrations, (b) SPSDVB-(PPV/PAA/DAR/PAA)$_n$ spheres and (c) SPSDVB-(PPV/PSS/DAR/PSS)$_n$. The excitation was set at 488 nm, emission was received from 527 ± 16 nm channel.

PAA or PSS (repeated for n times) followed by thermal treatment. It is to be noted that when tetralayer number n is 0.25 or 1.25, pre-PPV or PPV is the outmost layer; when n is 0.5, 1, 1.5, or 2, PAA or PSS is the outmost layer; and when n is 0.75 or 1.75, DAR is the outmost layer. The same meaning stands for the "n" in the following text. The emission spectra for these spheres are shown in Figure S5 in the Supporting Information. Generally, all the spheres gave broad emission in 425−650 nm. The addition of the first PAA layer resulted in the slight blue-shift of the PPV emission and the addition of the first PSS layer resulted in the slight red-shift of the PPV emission. Such blue-shift or red-shift can be attributed to the reduction or extension of the π conjugation of the coated layers. As mentioned above, the addition of DAR also red-shifted the spectra to some degree. However, when more layers were coated, the influence on the emission wavelength became smaller.

The emission intensities from flow cytometry measurements for these spheres are also shown in Figure 2. Several phenomena can be observed. First, the addition of PAA onto the PPV layer, always quenched the fluorescence of PPV, whereas the addition of PSS onto the PPV layer always enhanced the emission. Second, the addition of DAR onto the polyainon layer quenched the emission in both systems. Third, the addition of polyanions onto the DAR layer increased the emission in both systems. Fourth, the variation in the emission intensity of the second tetralayer (PPV/PAA/DAR/PAA or PPV/PSS/DAR/PSS) almost repeated that of the corresponding first tetralayer in both cases. The reason for the PAA-induced fluorescence quenching can be mainly attributed to the chain interpenetration between PAA and pre-PPV, and the reason for the PSS induced fluorescence enhancement can be mainly attributed to the PPV chain rigidification because of the presence of relatively rigid PSS. The quenching effect from DAR has been discussed previously during the optimization of [DAR]. Interestingly, the emission of the spheres increased again when coating PAA or PSS onto the DAR layer. There are two possible reasons for this phenomenon. First, some of DAR was "pulled off" by the polyanion in the dipping solution during the coating process. Second, with more layer of polyanion contacting the DAR layer, more cross-linking reaction might take place during thermal elimination and thus the amount of azo groups decreased. Therefore, the fluorescence quenching effect might be reduced.

After optimization, we decided to prepare the multilayer coated microspheres by consecutively coating pre-PPV, PSS, DAR, and PAA onto the substrate SPSDVB spheres followed by thermal treatment. The selection of polyelectrolytes and the arrangement for coating sequence were based on the above experimental results, aiming to obtain microspheres with relatively strong emission and surface functionality for bioconjugation. PSS was deposited in direct contact with PPV to enhance the emission, and PAA was deposited as the outmost layer to provide the desired functionality. DAR was used to form cross-linking sites with PSS and PAA.

2.2. Preparations and Characterizations of SPSDVB-(PPV/PSS/DAR/PAA) Microspheres. SPSDVB-(PPV/PSS/DAR/PAA) spheres were prepared after above optimization. Detailed investigation about the properties of these spheres was carried out, to examine if these spheres can provide desired properties required by practical applications. For comparison, some properties of the whole series of SPSDVB-(PPV/PSS/DAR/PAA)$_n$ spheres, were also examined. Here the tetralayer number n = 0.25, 0.5, 0.75, or 1 refers to spheres with PPV, PSS, DAR, or PAA as the outmost layer, respectively.

The digital photographs for these spheres with different number of coated layers are shown in Figure 3a. Compared to the appearance as white solids for all spheres before thermal elimination (Figure S6 in the Supporting Information), the spheres after thermal elimination displayed different colors under normal light, bright yellow for the spheres without DAR layer and dark yellow-greenish when having the DAR layer. The different apparent colors displayed are consistent with their UV−vis spectra (Figure S7, and more related discussion in Supporting Information). However, under the irradiation of 365 nm UV light, all the spheres emitted green fluorescence, but it is very obvious that the spheres with PSS as the outmost

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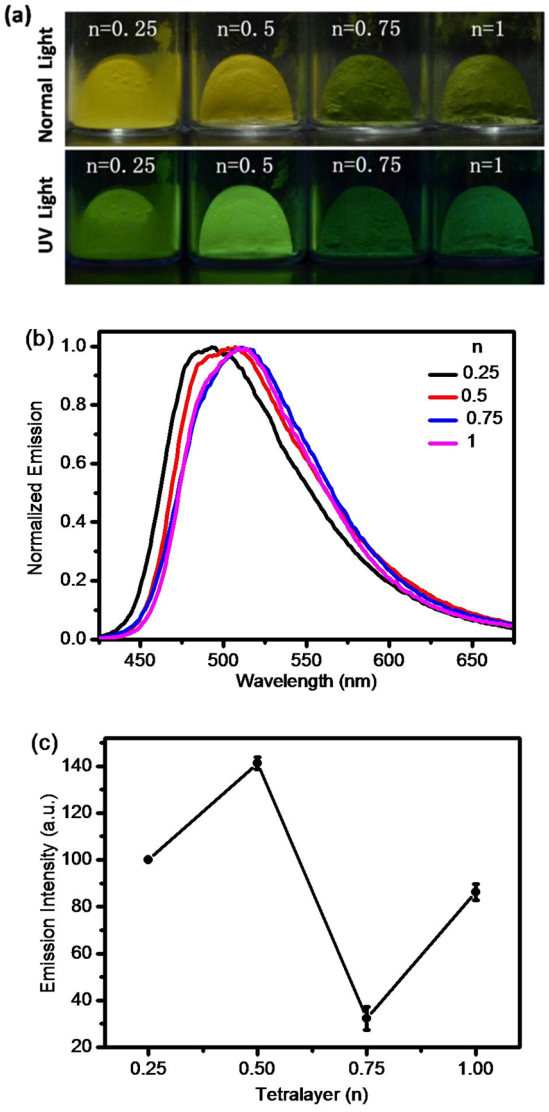

Figure 3. (a) Digital photographs spheres under normal light or 365 nm UV light. (b) Normalized fluorescence spectra (excited at 385 nm) and (c) emission intensities from flow cytometry (excited at 488 nm, emission received from 527 ± 16 nm channel) for SPSDVB-(PPV/PSS/DAR/PAA)$_n$ spheres. The error bars shown are based on the calculated standard deviations from the average value of three measurements from flow cytometry with spheres having the same composition but from different batches; for each measurement, the intensity was normalized with respect to the intensity of SPSDVB-PPV spheres to make the data from different measurements comparable.

layer had the strongest emission. The emission for the spheres with DAR layer was relatively weaker.

The normalized emission spectra from the regular spectrofluorometer and the emission intensity from flow cytometry are also shown in Figure 3. As expected, when PSS was the outmost layer and in direct contact with PPV, the fluorescence emission wavelength had a red shift about 15 nm, and the fluorescence intensity was greatly enhanced. After DAR was coated, the fluorescence emission wavelength remained unchanged, but the intensity was greatly quenched. After the introduction of PAA, the fluorescence emission wavelength remained unchanged, but the fluorescence intensity returned to the similar level as that of the initial SPSDVB-PPV spheres. The information from the photophysical study is generally consistent with the direct view of these spheres as shown above.

Therefore, on the basis of the above study, SPSDVB-(PPV/PSS/DAR/PAA), as the optimized composition for the spheres, should meet the requirements from the proposed applications in the introduction, with a compromise between the fluorescent intensity and the surface functionality. Microscopic studies were carried out on these spheres. As seen from the SEM and confocal fluorescent images (Figure 4), SPSDVB-(PPV/PSS/DAR/PAA) microspheres are uniform in size, morphology, and fluorescence emission, displaying a clear core−shell structure with tiny porosity. Such observation suggested that the whole LBL process was well-controlled.

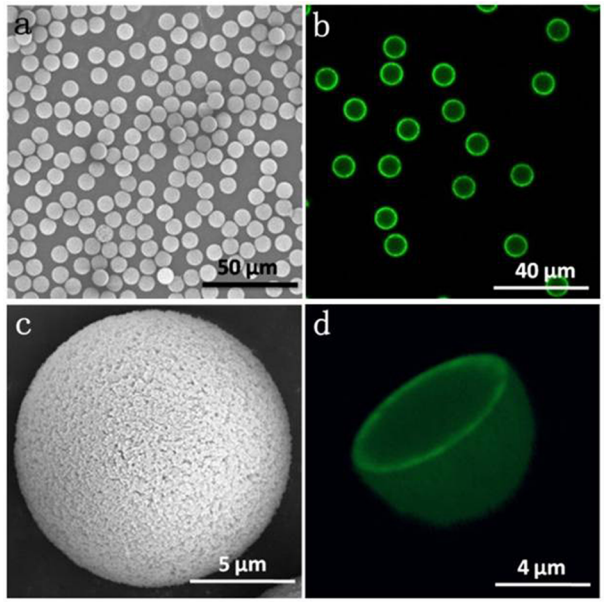

Figure 4. (a) SEM image and (b) the confocal fluorescent image from optical sectioning of SPSDVB-(PPV/PSS/DAR/PAA) microspheres; (c) SEM image for a single sphere, and (d) the 3D cross-section reconstruction image of the confocal data from the optical sectioning from the apex to the equator plane of one sphere. The confocal images were obtained by receiving emission at the 415−600 nm channel with excitation at 405 nm.

2.3. Characterization of the Cross-Linking Reaction and Solvent-Resistance Study. The introduction of DAR layer is to increase the stability of the self-assembled layers due to the formation of the cross-linking sites. According to the chemical reaction scheme (Scheme S1 in the Supporting Information), the nitrogen content should greatly decrease after the cross-linking due to the release of N_2 gas. The elemental analysis of nitrogen was carried out on SPSDVB-(pre-PPV/PSS/DAR), SPSDVB-(pre-PPV/PSS/DAR/PAA), SPSDVB-(PPV/PSS/DAR), and SPSDVB-(PPV/PSS/DAR/PAA) microspheres and the results are shown in Table S1 in the Supporting Information. There are two obvious facts that can be drawn from the data. First, for both spheres with DAR or PAA as the outmost layer, the nitrogen content before thermal elimination is much larger than that after elimination, which was consistent with the happening of the cross-linking reaction during heating. Second, for both spheres before and after thermal treatment, the addition of PAA layer onto the spheres reduced the nitrogen content. This also suggested that desorption of polycation (DAR) from the spheres when the

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spheres were dispersed in a polyanion (PAA) solution. One major concern about LBL method is the more or less desorption of the adsorbed layers in different environments, because the electrostatic interaction is relatively easy to destroy in many instances. After heating, covalent bonds formed between DAR and PAA or PSS to give stable sulfonates or carboxylates, and thus improved the stability. To demonstrate the improvement in the solvent resistance, we carried out the washing of the microspheres by different solvents. The emission intensities of the spheres measured by flow cytometry before and after washing were compared, and the emission spectra of the different washing solutions (supernatants) after washing were also compared.

The solvent-resistance study was first carried out by dispersing the SPSDVB-(PPV/PSS/DAR/PAA)$_n$ spheres into different organic solvents, such as methanol, toluene and chloroform for 8 h oscillation. Then the spheres and the supernatant were separated by centrifugation. The washed spheres were dried. The emission intensities of the microspheres were obtained by flow cytometry and the emission spectra of the supernatants were measured on a spectrofluorometer (Figure S8 in the Supporting Information). Most spheres had very similar emission intensity as those corresponding spheres without washing. In the case of using chloroform and toluene for washing, only the spheres with single PPV layer displayed slight decrease in the intensity upon washing, while intensity of other spheres remained almost unchanged. The emission intensity from the supernatants were consistent with the above emission intensity from the spheres. To ensure the emission intensities were comparable, we used exactly the same volume of solvents, and all the washing steps were strictly controlled. In the case of methanol, all the emission spectra of the supernatants displayed very large signal-to-noise ratio and low intensity, indicating that the PPV chains on the spheres were not easily washed off by methanol. In the case of chloroform and toluene, for spheres with DAR layer for cross-linking, the emission intensity of supernatants was also low, indicating that PPV chains on these spheres also have good resistance against chloroform and toluene. For those spheres without a DAR layer, there was more or less emission increase in the supernatants. Therefore, without the cross-linking, especially for spheres with a single PPV layer coating, the PPV chains were slightly washed off by toluene and chloroform. However, all the cross-linked multilayer-coated PPV fluorescent microspheres displayed good resistance against common organic solvents.

Considering the applications of fluorescent microspheres are usually carried out in aqueous environment and may involve solution with high ionic strength, further investigation into the fluorescence stability of the microspheres was carried out by washing the microspheres using water or a ternary mixture of H_2O-dimethylformamide-$ZnCl_2$ (3:5:2, w/w/w). This ternary mixture, here we call it as "dissociation agent", has been well-known for destroying the polyelectrolyte complexes by dissociating the ionic/hydrogen bonds linking the multilayers to the solid surface.[35] The washing and separation steps were carried out exactly the same as described above for organic solvents. For comparing the dissociation effect, we carried out the fluorescence study on two series of SPSDVB-(PPV/PSS/DAR/PAA)$_n$. Starting with the same SPSDVB-(pre-PPV/PSS/DAR/PAA)$_n$, one series of spheres were first thermally treated to give SPSDVB-(PPV/PSS/DAR/PAA)$_n$ and then washed by the "dissociation agent" ("washing after heating"); another series of spheres were first washed by the "dissociation agent" and then thermally treated to give the final SPSDVB-(PPV/PSS/DAR/PAA)$_n$ ("washing before heating"). The supernatants obtained after washing SPSDVB-(pre-PPV/PSS/DAR/PAA)$_n$ were thermally treated at 80 °C for 1 h to convert pre-PPV to PPV in solution. The emission intensities of the microspheres and the emission spectra of the supernatants are shown in Figure S9 in the Supporting Information. Detailed discussion about the information from Figure S9 was carried out in the Supporting Information.

To give a much clearer view about the above results from fluorescence stability in aqueous environment, further analysis of the directly obtained data in Figure S9 in the Supporting Information was carried out. The fluorescence remaining (%) of spheres after washing is shown in Figure 5a, by comparing the emission intensity of the washed spheres with that of spheres having same composition but no washing, based on the

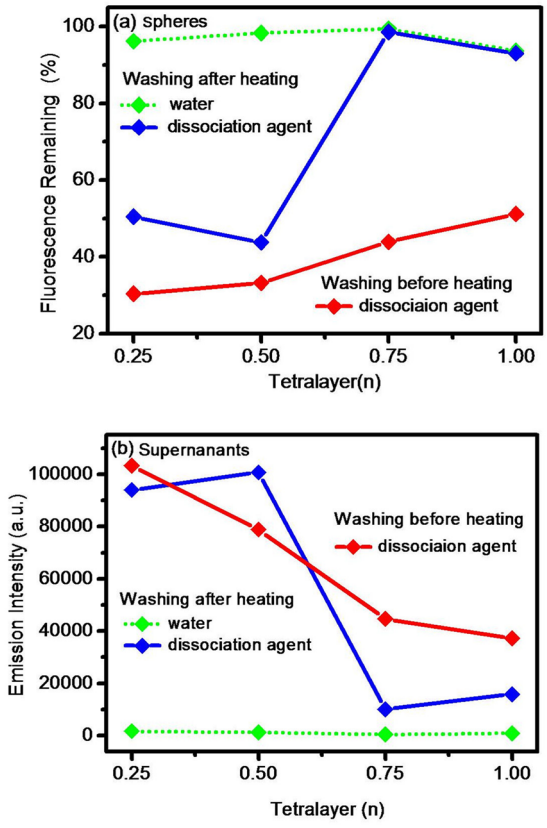

Figure 5. (a) Percentage of fluorescence remaining for the spheres washed by water or "dissociation agent" compared to the spheres from same batch but not washed, based on the emission intensity measured by flow cytometry for SPSDVB-(PPV/PSS/DAR/PAA)$_n$ spheres (excited at 488 nm, emission received from 527 ± 16 nm channel); (b) fluorescence intensity at the emission peak of the supernatants after washing the spheres. The supernatants from washing SPSDVB-(pre-PPV/PSS/DAR/PAA)$_n$ spheres were thermal treated to convert pre-PPV to PPV in solution before fluorescence measurement. "Washing after heating" refers to the SPSDVB-(PPV/PSS/DAR/PAA)$_n$ spheres were obtained with a normal step and then were washed; "Washing before heating" refers to SPSDVB-(PPV/PSS/DAR/PAA)$_n$ spheres were obtained with thermal treatment on the first washed SPSDVB-(pre-PPV/PSS/DAR/PAA)$_n$ spheres. The tetralayer number n = 0.25, 0.5, 0.75, or 1 refers to the spheres having pre-PPV (or PPV), PSS, DAR, or PAA as the outermost layer.

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data from flow cytometry in Figure S9a in the Supporting Information. The fluorescence intensity at the emission peak of the supernatants after washing the spheres is shown in Figure 5b. The information shown in panels a and b in Figure 5 is very consistent with each other. Washing by water had little influence on the emission intensity for the regularly obtained SPSDVB-(PPV/PSS/DAR/PAA)$_n$ spheres because the fluorescence remaining for the whole series was higher than 93% after washing; and negligible fluorescence was observed in the supernatants. In the case of using "dissociation agent", for those SPSDVB-(PPV/PSS/DAR/PAA)$_n$ obtained regularly, the fluorescence remaining was high (>93%) for the spheres with DAR layer to form cross-linking sites, but relatively low (<51%) for spheres without DAR layer; and correspondingly the emission intensity of the supernatants displayed the opposite trends. Note that the spheres without DAR still had about 50% of fluorescence remaining against washing by "dissociation agent", suggesting that the PPV without side groups also provides a certain fluorescence stability against washing. However, the fluorescence stability of the spheres against "dissociation agent" was further enhanced greatly by cross-linking reaction. For those spheres obtained with "washing before heating" procedure, the fluorescence remaining for the whole series of spheres was relatively low and the emission intensity of the supernatants was relatively high. Such observation is reasonable because washing was carried out before the cross-linking reaction took place. In addition, coating more layers onto the pre-PPV layer also provided some protection from washing off the inner pre-PPV layer. In all, the above study further confirms the cross-linking of the multilayers endowed spheres with a strong fluorescence stability in an aqueous environment, even with high ionic strength.

2.4. Characterization of the Surface Functionality and Bioconjugation Study. The presence of a carboxyl group on the surface of spheres is very important for using them in biorelated applications. In FTIR spectra for the SPSDVB-(PPV/PSS/DAR/PAA)$_n$ spheres (Figure S10 in the Supporting Information), only the spheres with PAA as the outmost layer have a peak around 1720 cm^{-1}, which is a typical peak for carboxyl group. The peak remained almost unchanged in the wavenumber and intensity even after being washed with the "dissociation agent". Therefore, the carboxyl groups have been successfully introduced on the surface of the spheres and should be stable on the spheres even in an aqueous environment with high ionic strength.

To demonstrate that such surface functionality is ready for bioconjugation in practical applications, the interaction between the fluorescent microspheres and biomolecules was investigated based on a systematic study using a regular spectrofluorometer. Bovine serum albumin (BSA) is a typical biomolecule with amino groups, which can interact with carboxyl groups. To make the detection of biomolecules loaded onto the microspheres possible, we used the TRITC (a commercial dye, structure shown in Figure S11 in the Supporting Information) labeled BSA (BSA-TRITC) in this study as the representative biomolecule. The fluorescence study was carried out in several steps. First, the emission spectra of the SPSDVB-(PPV/PSS/DAR/PAA) spheres were dispersed in phosphate buffer solution (PBS pH 7.4) and BSA-TRITC was dissolved in PBS were compared. As shown in Figure 6a, the two spectra had little overlap. The emission from the spheres was among 470−600 nm with a peak around 514 nm, whereas TRITC molecules gave emission among 560−675 nm with a peak around 592 nm. Second, the SPSDVB-(PPV/PSS/DAR/PAA) spheres were directly mixed with BSA-TRITC, or reacted with BSA-TRITC using EDC/NHS (a commonly used coupling agent for biomolecules) in PBS. As shown in Figure 6b, both spheres displayed significant emission at 592 nm, indicating both methods successfully introduced BSA onto the spheres. The coupling method seems to be more efficient because much higher fluorescence emission at 592 nm was observed, using the emission from spheres themselves as the inner standard. Third, the fluorescence measurement was also carried out on the spheres after directly mixing TRITC with SPSDVB-(PPV/PSS/DAR/PAA) spheres (SPSDVB-(PPV/

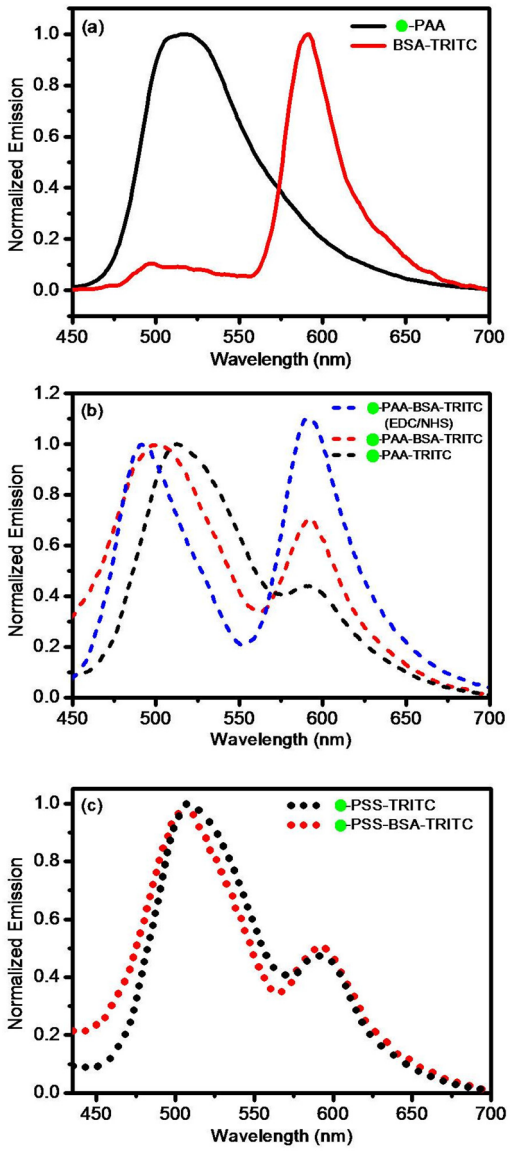

Figure 6. Normalized emission spectra of (a) BSA-TRITC and SPSDVB-(PPV/PSS/DAR/PAA) spheres; (b) SPSDVB-(PPV/PSS/DAR/PAA)-BSA-TRITC, SPSDVB-(PPV/PSS/DAR/PAA)-BSA-TRITC (EDC/NHS), and SPSDVB-(PPV/PSS/DAR/PAA)-TRITC microspheres ; (c) SPSDVB-(PPV/PSS/DAR/PSS)-BSA-TRITC and SPSDVB-(PPV/PSS/DAR/PSS)-TRITC. BSA-TRITC was dissolved in PBS and the spheres were dispersed in PBS during the measurement. In the figures, the green dot represents the inner part of the spheres, SPSDVB-(PPV/PSS/DAR), and the outer layers (PAA or PSS, and TRITC or BSA-TRITC) are directly marked out. The excitation was set at 385 nm.

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PSS/DAR/PAA)-TRITC). For control, the concentration of TRITC solution was adjusted to give the same emission intensity as the above that for BSA-TRITC solution, and the volumes of the two solutions used for mixing with spheres were the same. As also shown in Figure 6b, very low emission intensity at 592 nm was observed for SPSDVB-(PPV/PSS/DAR/PAA)-TRITC spheres, suggested that the relatively strong emission of SPSDVB-(PPV/PSS/DAR/PAA)-BSA-TRITC and the SPSDVB-(PPV/PSS/DAR/PAA)-BSA-TRITC (EDC/NHS) are mainly due to the interaction between BSA molecules and spheres, instead of the interaction between TRITC and the spheres. Fourth, to rule out the suspicion about the possible direct adsorption of BSA-TRITC macromolecules onto the spheres without need of carboxyl groups, the SPSDVB-(PPV/PSS/DAR/PSS) spheres were directly mixed with BSA-TRITC and TRITC, respectively (Figure 6c). However, the emission at 592 nm for SPSDVB-(PPV/PSS/DAR/PSS)-BSA-TRITC and SPSDVB-(PPV/PSS/DAR/PSS)-TRITC were both as low as that for SPSDVB-(PPV/PSS/DAR/PAA)-TRITC. This result further confirmed that the carboxyl group is indispensable in interaction with biomolecules. Interestingly, the emission peak for PPV on the spheres kept blue-shifting when the emission intensity of TRITC increased, comparing the spectra from different spheres. It seems that loading of other molecules onto the spheres could interrupt the conjugated length of PPV system but it needs further investigation for more accurate explanation. In addition, the FTIR study also gave some auxiliary proof for the successful bioconjugation reaction (Figure S12 and the related discussion in Supporting Information). In all, a conclusion can be made at this point is that direct adsorption or nonspecific interaction can only introduce very few BSA-TRITC or TRITC molecules onto the spheres; and the presence of −COOH groups greatly facilitated the interaction with biomolecules.

2.5. Stability Study. The thermal stability and photostability of fluorescent spheres were important performance indexes for practical applications. The photostability study was performed on SPSDVB-(PPV/PSS/DAR/PAA) spheres by measuring the emission spectra using a regular spectrofluorometer. As shown in Figure S13 in the Supporting Information, the intensity of the emission maximum remained 89% of the initial intensity after 10 min of irradiation, and 71% after 1 h. The TGA measurements were carried out for whole series of SPSDVB-(PPV/PSS/DAR/PAA)$_n$ spheres (Figure S14 in the Supporting Information). Coating PSS, DAR, and PAA onto the PPV layer slightly improved the thermal stability of the spheres by increasing the degradation temperature from 400 to 450 °C. Such thermal stability and photostability should be sufficient for the general applications of these fluorescent spheres, which are usually carried out around room temperature with a very short excitation time.

3. CONCLUSION

The cross-linked multilayer-coated fluorescent polymer microspheres were successfully prepared using LBL technique followed by thermal treatment. On the basis of the characterizations from regular spectrofluorometer, flow cytometry, and microscopy, the preparative conditions and the composition of the different layers were optimized. SPSDVB-(PPV/PSS/DAR/PAA) spheres were prepared to provide relatively strong fluorescence and surface reactive sites for bioconjugation. Microscopic study demonstrated that the SPSDVB-(PPV/PSS/DAR/PAA) spheres have uniform size and even fluorescence emission with a clear core−shell structure. The cross-linked multilayer coated spheres were found to have very good resistance against solvents. The fluorescence and carboxyl functionality are stable against solvent washing, even in the case of using "dissociation agent". The feasibility for bioconjugation was also successfully demonstrated for these spheres. These spheres were found to have good thermal stability and photostability. In all, the cross-linked multilayer not only protected the inner PPV layer from fluorescence loss but also provided the surface functionality for effective bioconjugation. We believe this preparative strategy can be extended to preparing various fluorescent microspheres with substrate spheres having different size and chemical compositions, to meet the vast demanding from the practical applications.

■ ASSOCIATED CONTENT

Supporting Information

The experimental section, cross-linking reaction scheme, chemical structure of TRITC dye, elemental analysis data, UV−vis absorption spectra, FTIR spectra, TGA curves, additional SEM and fluorescence microscopic images, photographs, and emission spectra, as well as some related discussion. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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