Hydrogel-based microbeads for Raman-encoded suspension array using the reversed-phase suspension polymerization method and ultraviolet light curing

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Abstract

A one-step synthesis using the reversed-phase suspension polymerization method and ultraviolet light curing is proposed for preparing the Raman-encoded suspension array (SA). The encoded microcarriers are prepared by doping the Raman reporter molecules into an aqueous phase, and then dispersing the aqueous phase in an oil phase and curing by ultraviolet light irradiation. The multiplexed biomolecule detection and various concentration experiments confirm the qualitative and quantitative analysis capabilities of the Raman-encoded SA with a limit of detection of 52.68 pM. The narrow bandwidth of the Raman spectrum can achieve a large number of codes in the available spectral range and the independence between the encoding channel and the fluorescent label channel provides the encoding method with high accuracy. This preparation method is simple and easy to operate, low in cost, and high in efficiency. A large number of hydrogel-based encoding microbeads could be quickly obtained with good biocompatibility. Most importantly, concentrating plenty of Raman reporter molecules inside the microbeads increases the signal intensity and means the molecular assembly is not limited by the functional groups; thus, the types of materials available for Raman encoding method are expanded. Furthermore, the signal intensity–related encoding method is verified by doping different proportions of Raman reporter molecules with our proposed synthesis method, which further increases the detection throughput of Raman-encoded SA.

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Introduction

In life science field, multiplexed detection technology enables simultaneous obtainment of multiple target signal in one rare sample [1–4]. Suspension array (SA) has been considered as one of the most attractive multiplexed detection methods due to its good performance in limit of detection and efficiency [5–7]. Correct identification of microcarriers is the crucial part of SA to achieve the simultaneous detection of multiple molecules, and it highly relies on the encoding method of microcarriers [8–10]. Optics-encoded microbeads play an important role in SA-detecting platforms [11], among which fluorescence encoding methods are most commonly used [12–14]. Since the fluorescence spectrum has a broad spectral peak, there are limited number of materials that can be used to encode in the available spectral range [15, 16]. In addition, fluorescent label is also required in the quantitative analysis process and fluorescence resonance energy transfer exists between the multi-color fluorescent materials, thereby affecting the accuracy of detection [17, 18].

Raman spectrum, also known as fingerprint spectrum, provides stable peak position for substance with different functional groups [19, 20]. The specific spectral peaks for different molecules could be used to encode the microcarriers of SA [21–23]. Compared with the fluorescence encoding method, SA encoded with Raman spectra can achieve more encoding quantities in the available spectral range because of the narrow bandwidth [24, 25]. More importantly, since Raman spectra are used for encoding and fluorescent label is used for quantitative detection, the two independent channels could ensure accuracy for the encoding method.

Raman scattering signal is weak as only one millionth of the intensity of the incident light and the detection of its signal usually count on the enhancement caused by noble nanoparticles [10, 26, 27]. Considering that silver is very susceptible to oxidation in air, gold nanoparticles, because of its good stability, are commonly used as substrates in Raman scattering signal enhancement. In practical application, we need to prepare the gold nanoparticles with uniform size to produce a uniform signal enhancement. However, the preparation of gold nanoparticles increases the costs, and requires complex processes and sophisticated synthesis techniques. After preparation, the assembly of the molecule with gold nanoparticle typically relies on the thiol group of the molecule, which could combine with the gold nanoparticle via Au-S bond [28, 29]. It means that the Raman reporter molecules we chose should have the characteristic peaks that do not overlap with each other [25, 30] and contain a thiol group for molecular assembly. These requirements make the encoding method complicated and the available encoding materials are limited. In addition, it is inevitable that different sizes of gold nanoparticles are produced by the limitation of the synthesis process, and the distances and arrangements between the gold nanoparticles would cause different degrees of enhancement of the Raman scattering signal [31, 32]. Therefore, the enhanced Raman scattering signal is not stable, and it is difficult to use the signal intensity as another encoding dimension. In summary, method encoded with the enhanced Raman scattering signal is limited in the number of codes [24, 25, 33].

In this paper, we provide a one-step synthesis of Raman-encoded microbeads (REBs) using suspension polymerization method and ultraviolet light curing. The REBs, which were prepared with a low-cost and large-scale manner, acted as the microcarriers of SA for detecting multiple biomolecules. Reversed-phase suspension polymerization method is a common method for the preparation of microbeads [34, 35], and ultraviolet light curing is a photochemical reaction often used in industry. Raman reporter molecules as the encoding materials are uniformly mixed in an aqueous phase solution, then the doped aqueous phase is cut into small droplets in the oil phase under continuous stirring, followed by photocuring by irradiation with ultraviolet light. A large number of REBs with similar size can be quickly attained by screening process.

After surface modification, various biomolecules as the probes are grafted with different kinds of REBs to achieve simultaneous detection of multiple target biomolecules in a single sample. This method reduces the cost and simplifies the preparation process of Raman-encoded SA. Since the assembly of Raman reporter molecules is not limited with the functional group, more types of encoding materials can be used. With the stable signal, different proportions of Raman reporter molecules could be doped to encode the microcarriers of SA with our proposed synthesis method, thus further expanding the encoding capacity. Moreover, since polyethylene glycol diacrylate (PEGDA) is used as the aqueous phase, it does not release acidic products during their degradation [36], and the synthetic encoding microbeads have better biocompatibility than the traditional microcarriers of polystyrene microbeads [37, 38].

Experimental section

Reagents and materials

4-Methylbenzenethiol (4-MT), 2-naphthalenethiol (2-NT), 4-hydroxybenzenethiol (4-HBT), rhodamine 6G (R6G), β-carotene, 3-methoxybenzenethiol (3-MBT), bovine serum albumin (BSA), and dopamine hydrochloride were purchased from Aladdin Industrial Corporation, China. 2-Hydroxy-4′-methylpropiophenone-(2-hydroxyethoxy)-2-methylpropiophenone (photoinitiator) was purchased from Macklin reagent, China. PEGDA (average MW = 250), hexadecane, and EM-90 were purchased from Sigma-Aldrich. Three types of IgGs were purchased from Bioss Biotechnology, China. Three types of QDs-labeled anti-IgGs were purchased from Nanjing Technology Co., China. Phosphate buffer saline (PBS) was purchased from Beijing Solarbio Science & Technology Co., Ltd.

Apparatus

Raman spectra were obtained by a confocal Raman microscope (HORIBA LabRAM HR800) with a × 50 microscope objective. A 785 nm laser was focused on the sample with a power of 7.8 mW. The acquisition time was 30 s with 2 repetitions per spectrum. A ZEISS SUPRA® 55 was used to acquire the scanning electron microscopy (SEM) images with an accelerating voltage of 3 kV. Fourier-transform infrared (FT-IR) spectra were performed using a Fourier-transform infrared spectrometer (Thermo Scientific Nicolet iS 50). The measurements of fluorescent spectra were carried out on Spectrofluorophotometer (RF-5301PC, SHIMADZU) with 3 repetitions per spectrum. The fluorescence microscope images were attained by microscope (DM2500, Leica).

Synthesis of REBs

Reversed-phase suspension polymerization method and ultraviolet light curing were used to prepare the REBs. The disperse phase was prepared by mixing PEGDA, Raman reporter molecules, and photoinitiator. The continuous phase was composed of hexadecane and EM-90 with a ratio of 4:1. After mixing the above two solutions together, the disperse phase was dispersed into small droplets in the continuous phase under continuous stirring. Then, the stirring solution was exposed to the ultraviolet light to make the droplets solidify. To be more specific, 20 mg Raman reporter molecules and 20 mg photoinitiator were added in 1 mL PEGDA solution with vigorous stirring and ultrasonication for 15 min. Then, 3.2 mL hexadecane and 800 μL EM90 were mixed together and shaken until it formed a stable continuous phase. Next, 200 μL doped PEGDA solution was added into the continuous phase drop by drop in a 5-mL beaker with stirring. One minute later, the beaker was irradiated by an ultraviolet light with a power of 7 mW/(cm^2). For different kinds of Raman reporter molecules, the time of light curing differs from 1 to 10 h; for 4-MT, 2-NT, 4-HBT, R6G, 3-MBT, and 4-MT, the time of light curing is 1–2 h; for their mixture, the light curing time needs to be extended appropriately; for β-carotene, the time of light curing is 8–10 h. And the photopolymerization process can be observed under a microscope by taking out a small amount of liquid sample. Afterwards, the solidified beads were washed by cyclohexane and ethanol for three times, followed by the filtering by micro sieves. Finally, hydrogel-based REBs were obtained with the uniform sizes and dispersed in deionized water for next modification.

Surface modification of REBs and probe grafting

In this step, dopamine was utilized to provide REBs with functional groups. Then, IgGs as the probes were grafted onto the surface of REBs. After covering the excess functional groups with blocking solution, the Raman-encoded SA were prepared. The preparation details are as follows: First, 10 mg dopamine was dispersed in 5 mL Tris-HCl buffer solution (10 mM, pH = 8.5). Second, 10 mg REBs were added into the dopamine solution and stirred for 18 h at room temperature. Then, the excess dopamine was removed by washing with deionized water for five times and the modified REBs were redispersed in 1 mL phosphate buffer solution (PBS, 50 mM, pH 7.4). Finally, 100 μL of microbead dispersed solution was added into a mixed solution of 100 μL IgG solution (1 mg/mL), 200 μL EDC (10 mg/mL), and NHS (10 mg/mL) solution, and incubated for 2 h at 37 °C. After that, the probe-grafted REBs were washed by PBS solution for three times and the uncovered functional groups were blocked by 4 mL 1% BSA solution for 8 h at 4 °C.

Multiplexed protein detection and concentration response experiments

We performed multiplexed detection and concentration response experiments to verify the qualitative and quantitative analysis capabilities of the Raman-encoded SA for biomolecule detection.

In the multiplexed detection experiment, we used three kinds of REBs prepared by the above method to graft with three different IgGs, completing the mixed analyte detection of three different QDs-labeled anti-IgGs. Specifically, the three kinds of REBs were respectively encoded with 4-HBT, 2-NT, and a mixture of 2-NT and 3-MBT, then grafted with rabbit, mouse, and human IgG separately. The mixed analytes were composed of 525 nm QDs-labeled goat anti-rabbit IgG, 565 nm QDs-labeled goat anti-mouse IgG, and 605 nm QDs-labeled goat anti-human IgG. The three kinds of SAs were reacted with the mixed analytes under stir for 1 h at 37 °C. To remove the unreacted QDs-labeled anti-IgG solution, the reacted SAs were washed with PBS for five times, and then dispersed in PBS solution for further decoding and analysis.

For the concentration response experiment, we prepared the 2-NT-encoded REBs and grafted them with human IgG, then different concentrations of 605 nm QDs-labeled goat anti-human IgG solution were acted as analyte and tested by the prepared SA. The concentrations of the target analytes were 30.00 nM, 15.00 nM, 7.50 nM, 3.75 nM, 1.88 nM, 937.50 pM, 468.75 pM, 234.38 pM, 117.19 pM, and 58.59 pM, while 0 pM was used as a blank control group.

Results and discussion

Experimental principle

The synthesis and detection scheme of Raman-encoded SA is shown in Fig. 1. First, reversed-phase suspension polymerization method and ultraviolet light curing were used to prepare the REBs. The disperse phase was prepared by doping Raman reporter molecules and photoinitiator into PEGDA solution. The continuous phase was composed of hexadecane and EM-90. Then, the disperse phase was dispersed into small droplets in the continuous phase under continuous stirring, and the mixed solution was exposed to the ultraviolet light to solidify the droplets. A few hours later, the solidified microbeads were obtained and processed by washing. Next, filtering and collection were conducted to attain the REBs with similar size. Second, dopamine was utilized to modify the REBs with functional groups [39, 40], and then IgG as the bio-probe was grafted onto the surface of REBs. After that, the uncovered groups were blocked by BSA solution. Finally, the Raman-encoded SA was capable of recognizing the specific targets.

Fig. 1 The synthesis and detection scheme of Raman spectra encoded SA using reversed-phase suspension polymerization method and ultraviolet light curing
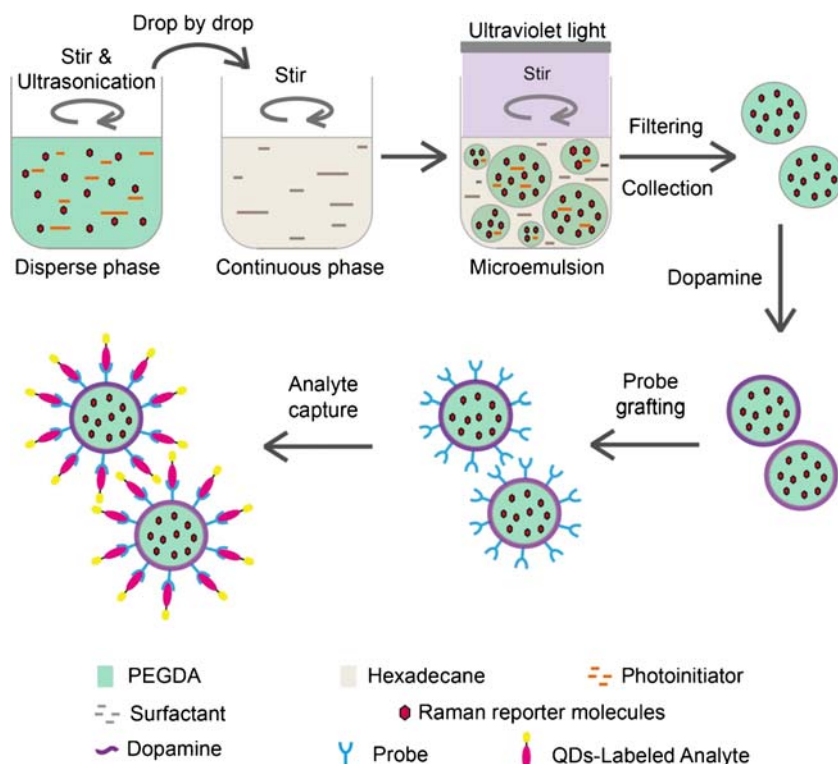

By using the above synthesis method, different kinds of Raman reporter molecules could be encapsulated into the microbeads. Without the restriction in functional group, there is no need to consider different methods or further modification for the assembly of specific encoding materials. Thus, any material with a unique Raman spectral peak can be used as the encoded Raman reporter molecules. In this paper, six kinds of Raman reporter molecules were taken as examples to verify the feasibility of our encoding method. The original Raman spectra and structural formulas are shown in Fig. 2. As we marked in the figure, 4-MT has the specific peak of 797 cm^{-1}, 2-NT is characterized by the peaks of 518 and 1383 cm^{-1}, 4-HBT holds the unique peak of 822 cm^{-1}, 610

and 1363 cm^{-1} are taken as the characteristic peaks of R6G, β-carotene is recognized by the peak of 1156 cm^{-1}, and 3-MBT is dominated by the peak of 994 cm^{-1}.

Characterization of REBs

The proposed Raman-encoded SA are available in two encoding methods. One is the use of intensity independent signal, which is encoded by the characteristic peaks of different Raman reporter molecules. The other is to dope the Raman reporter molecules with different ratios, that is, to encode with different signal intensities of the encoding materials. Theoretically, Raman spectra can achieve a lot number of

Fig. 2 The original Raman spectra (left) and structural formula (right) of 6 kinds of Raman reporter molecules. The shadow parts marked in the figure are the characteristic peaks used to encode
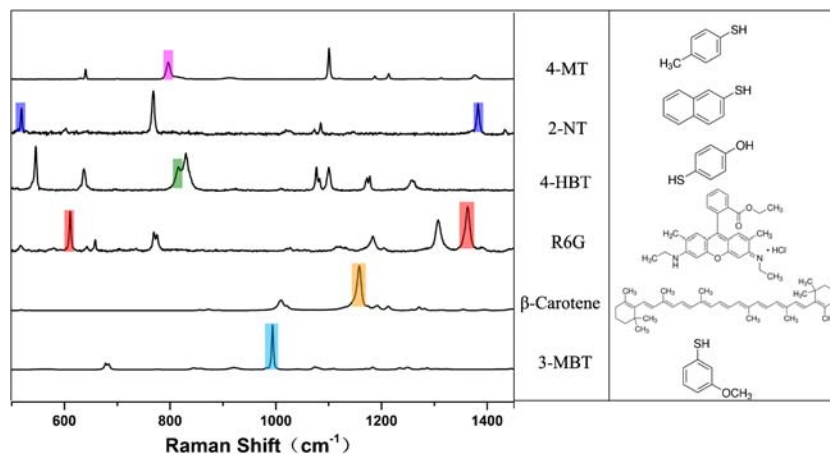
codes in the available spectral range due to its narrow spectral bandwidth, but many similar functional groups of molecules have spectral overlap problems, making the materials available for encoding limited and further affecting the number of codes. Similar to fluorescence encoding method, the number of codes can be further increased by encoding with signal intensity ratios of different encoding materials.

When the first method is applied for encoding, the encoded signal is unrelated with the intensity of Raman spectra. For making the encoding method more intuitive, the encoded Raman spectra are converted into a series of binary sequences according to the position of the characteristic peaks. Figures 3a, b, c, and d are the Raman spectra of microbeads encoded with one, two, three, and more than three kinds of encoding materials, respectively. The Raman spectrum of plain microbeads is shown at the top of Fig. 3a as a background signal. Comparing Fig. 3a with Fig. 2, it is found that six different kinds of Raman reporter molecules can be encapsulated separately into the microbeads and this process do not affect the encoding signal. Then, a variety of Raman reporter molecules were used for composite encoding. As we marked in the shadow parts of Fig. 3, all the characteristic peaks used

for encoding can be clearly seen and different Raman reporter molecules do not interfere with each other. The experimental results have a good signal-to-noise ratio, which confirms the feasibility of one-step synthesis for multiple kinds of Raman reporter molecule-encoded microbeads.

To verify the operability of signal intensity encoding method, we changed the amount of doped 3-MBT to control the intensity of the encoding peak. The experimental results are shown in Figs. 4a and b. As the number of 3-MBT molecules increases, the intensity of the Raman spectral peaks grows linearly. In order to enhance the credibility of this intensity-related encoding method, we carried out a random detection to 12 microbeads prepared with 30 μL of 3-MBT. As shown in Fig. 4c, the signal intensities of the 12 detected microbeads are slightly different, with a minimum of 196.332, a maximum of 214.420, and an average of 205.072, but their standard deviation (SD) is 6.337 and coefficient of variation (CV) is 3.09%, so the intensities are basically within the same gradient distribution interval. There is no case that the signal is too strong or weak and be recognized as the other encoding ratios. Thus, we can proportionally encode a variety of Raman reporter molecules by controlling the amount of doped Raman reporter
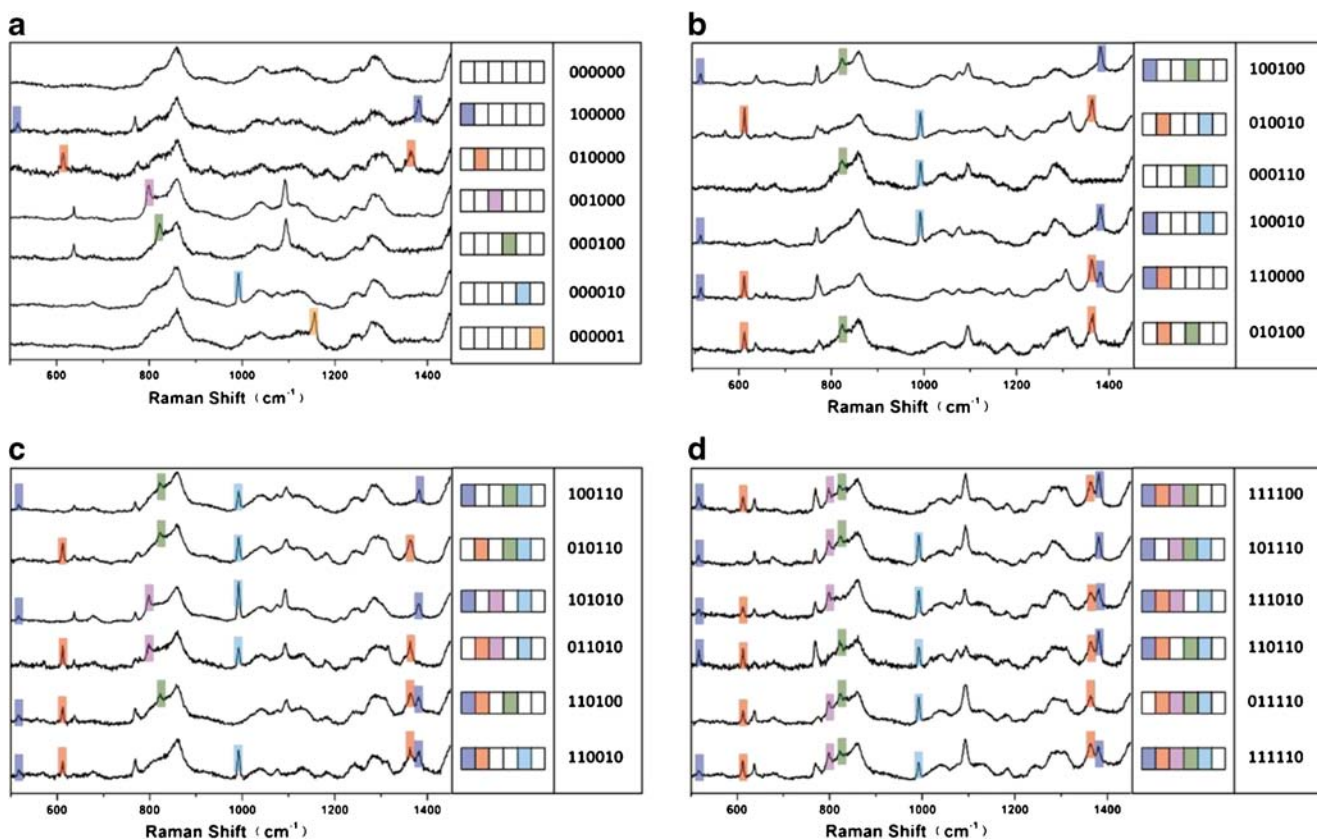

Fig. 3 Raman spectra of REBs. **a** Raman spectra (left) and binary sequences (right) of plain microbeads and microbeads encoded with one kind of Raman reporter molecules. **b** Raman spectra (left) and binary sequences (right) of microbeads encoded with two kinds of Raman reporter molecules. **c** Raman spectra (left) and binary sequences (right) of microbeads encoded with three kinds of Raman reporter molecules. **d** Raman spectra (left) and binary sequences (right) of microbeads encoded with more than three kinds of Raman reporter molecules. The shadow parts marked in the figure are the selected characteristic peaks
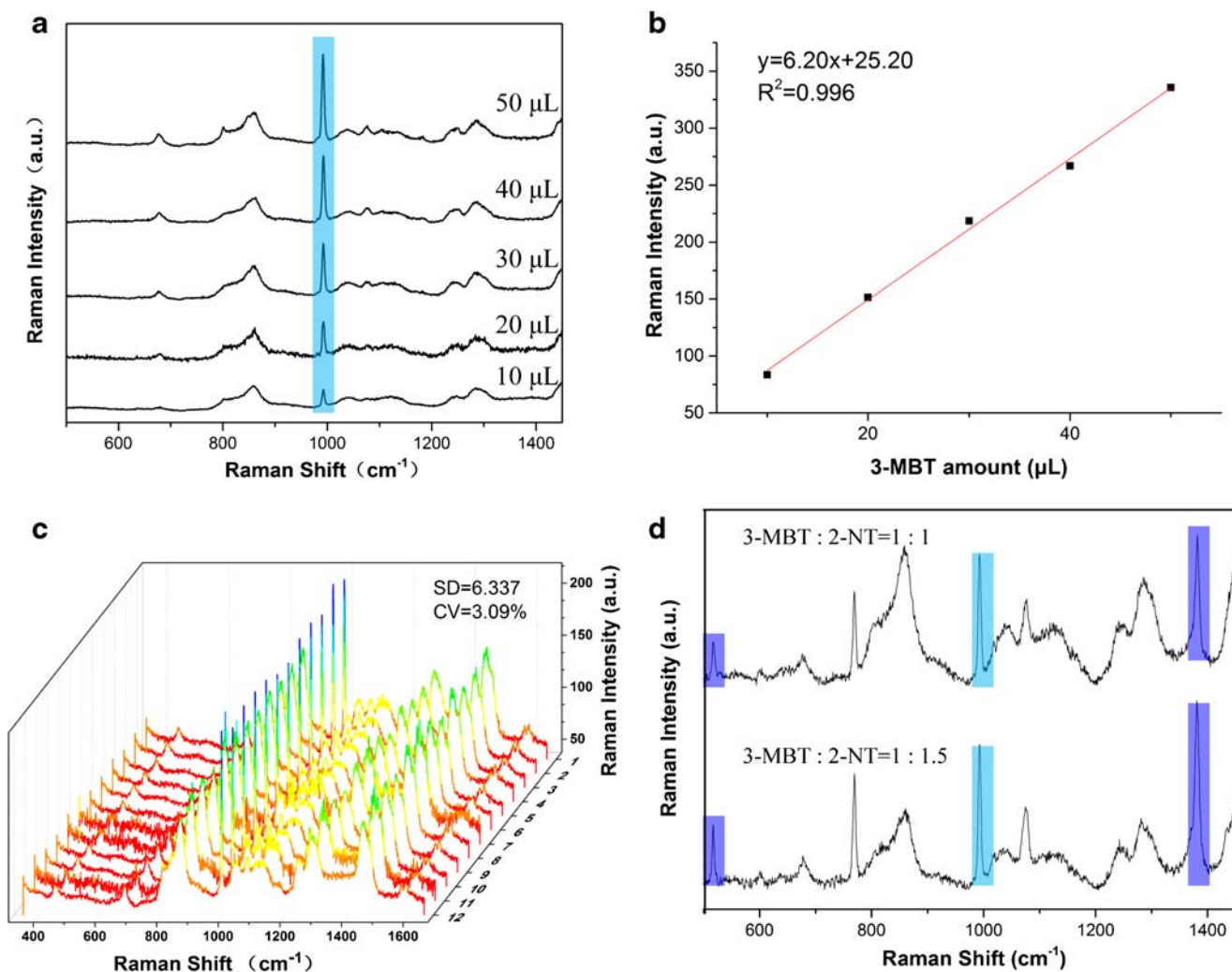
Fig. 4 Raman spectra of REBs encoded with different intensities of encoding signal. **a** Raman spectra of REBs encoded with 10 μL, 20 μL, 30 μL, 40 μL, and 50 μL of 3-MBT, respectively. **b** The fitted curve of Raman intensity of REBs and 3-MBT amounts. **c** Raman spectra of 12 random REBs encoded with 30 μL of 3-MBT. **d** Raman spectra of REBs encoded with 3-MBT and 2-NT in signal intensity ratios of 1:1 and 1:1.5, respectively

molecules. By doping 3-MBT and 2-NT with ratios of 1:1 and 1:1.5, respectively, we prepared two kinds of REBs with different signal intensity ratios, and the Raman spectra were measured as shown in Fig. 4d. The experimental results show that the intensities of the encoded characteristic peaks marked in the shadow are consistent with our encoding design. Therefore, it is reasonable to claim that the Raman spectra without signal enhancement has good stability and can be used for processing signal intensity encoding.

Characterization of surface-modified REBs

After synthesizing the microbeads by reversed-phase suspension polymerization method and ultraviolet light curing, we obtained the REBs with various sizes. Then, microsieves were used to attain the REBs with uniform size (Fig. 5a). The optical micrographs and size distribution diagrams of initial REBs and dopamine-modified REBs are shown in Fig. S1 in the Electronic Supplementary Material (ESM). It can be concluded that the size distribution of the initial microbeads is relatively uniform and the size is concentrated at 32 μm. And after being modified with dopamine, the size of REBs did not change significantly, remaining concentrated at 30–36 μm, which means that the change in the size of the REB by the dopamine layer is negligible. As Figs. 5b and c show, the initial REB has a round and smooth surface. Next, dopamine was applied to modify the surface of REBs for providing sufficient catechol/quinone groups [39]. The modified REBs are shown in Figs. 5d and e. Compared with the initial REBs, the rough surface of modified REBs demonstrates that dopamine has been successfully polymerized on the surface. And the FT-IR spectra of initial REBs and dopamine-modified REBs are shown in ESM Fig. S2, which proved that there are hydroxyl and quinone groups on the surface of
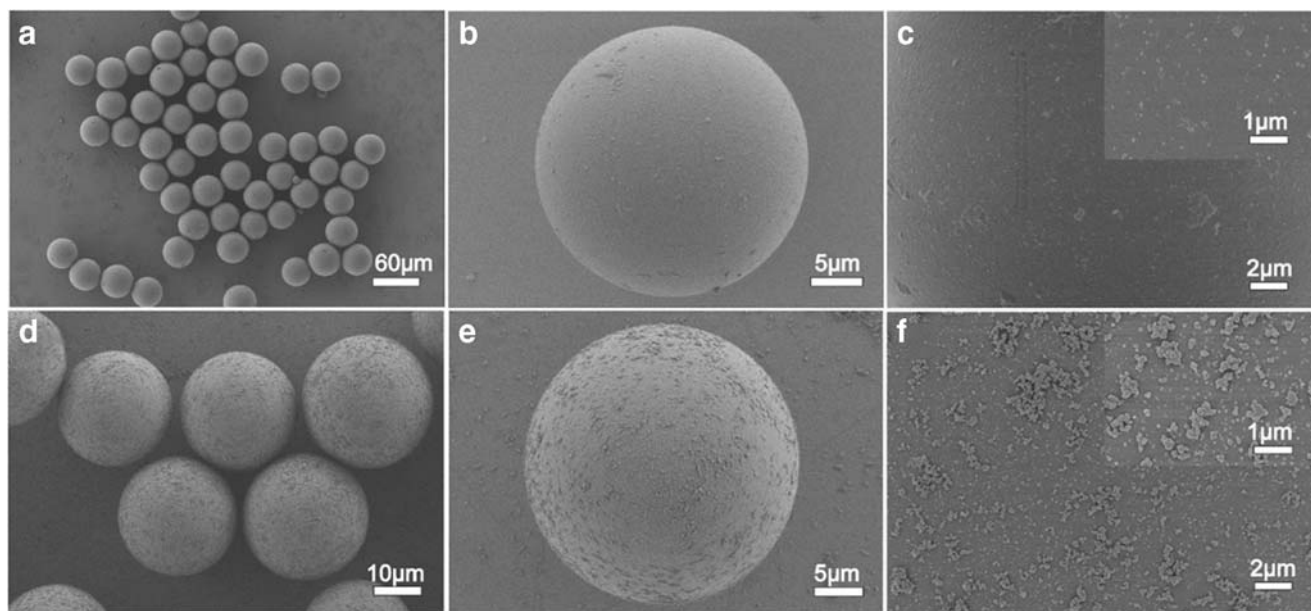
Fig. 5 SEM images of **a**–**c** initial REBs and **d**–**f** dopamine-modified REBs

dopamine-modified REBs and the number of carbonyl groups also increases.

Application of SA in multiplexed protein detection

To characterize the performance of Raman-encoded SA in multiplexed detection, we prepared three kinds of REBs encoded with 4-HBT, 2-NT, and a mixture of 2-NT and 3-MBT, which correspond to the binary sequences of 000100, 100000, and 100010. Then, the three kinds of REBs were grafted with rabbit, mouse, and human IgG respectively as probes to achieve the selective capture of 525 nm QDs-labeled goat anti-rabbit IgG, 565 nm QDs-labeled goat anti-mouse IgG, and 605 nm QDs-labeled goat anti-human IgG in a mixed sample.

The fluorescence image after the reaction is shown in Fig. 6a. With the excitation of 405 nm laser, different microbeads emitted three independent colors without interference between each other. It indicates that the REBs grafted with a specific probe can only selectively recognize one kind of analyte. The decoding results of the microbeads with different colors are shown in

Fig. 6b. The correlation between the encoding signal and the fluorescent label represents the relationship between the encoded microbeads and the target analyte. The above results indicate that the three kinds of REBs that grafted with different specific probes have selective binding with their target molecules. Hence, it should be noted that our Raman-encoded SA can be used to achieve simultaneous detection of multiple targets in a single sample.

To further demonstrate the quantitative ability of our method to biomolecules, we conducted a series of concentration response experiments. The prepared SA was encoded with 2-NT and grafted with human IgG. Ten groups of 605 nm QDs-labeled anti-human IgG with different concentrations were considered as the analytes. Figure 7a is the fluorescence spectra obtained from the 10 groups of concentration response experiments. As we reduce the concentrations of analyte, the fluorescence intensities gradually decrease. Taking the fluorescence intensity value at 605 nm as the ordinate and the analyte concentration value as the abscissa, we obtained a linear response curve by fitting these values in the detection

Fig. 6 The multiplexed protein detection. **a** Fluorescence image of three kinds of reacted SA. **b** The decoding Raman spectra of three kinds of reacted SA
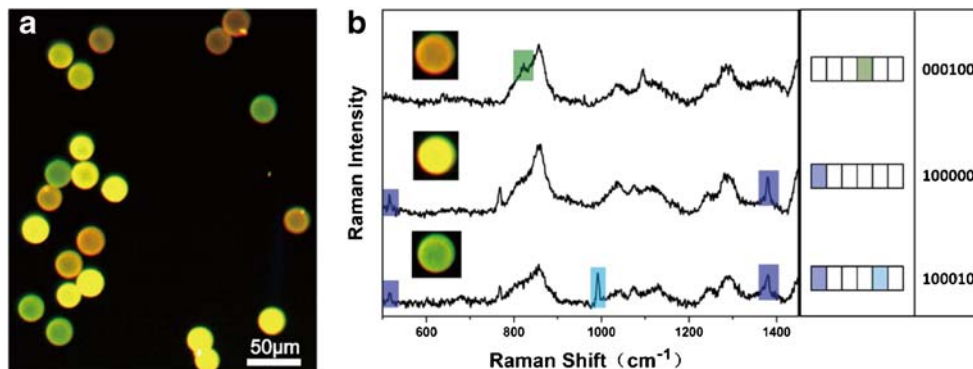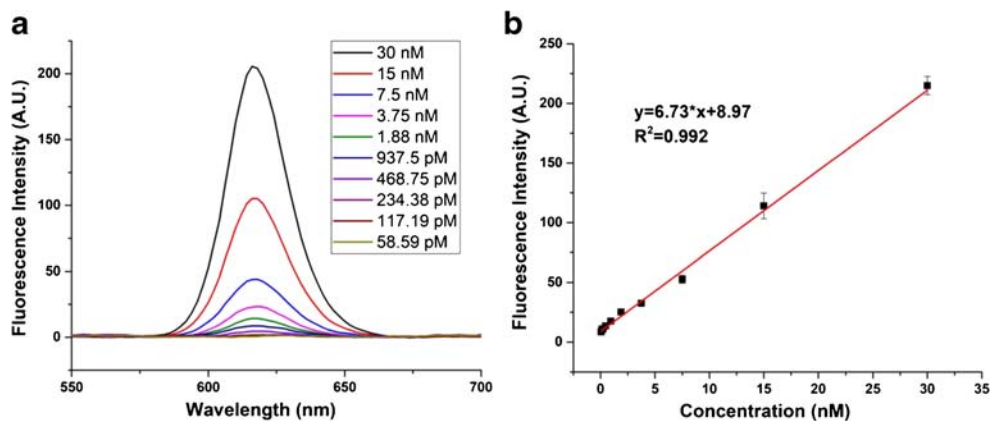
Fig. 7 a Fluorescence spectra of ten groups of reacted SA with different analyte concentrations. **b** The fitted concentration response curve

range from 58.59 pM to 30 nM (Fig. 7b). According to the fluorescence intensity value of the blank control group, the detection limitation of the experiment was calculated by the three-fold error method to be 52.68 pM. Since the detection limitation is related with the fluorescence intensity of the label, better performance can be achieved by using label QDs with higher quantum yields or increasing its binding efficiency with antibody [41]. Above experiments confirm that the Raman-encoded SA prepared by reversed-phase suspension polymerization method and ultraviolet light curing has good performance in quantitative analysis of biomolecules.

Conclusions

In summary, we propose a one-step synthesis of Raman-encoded SA using reversed-phase suspension polymerization method and ultraviolet light curing. Raman spectra as the encoding signal have narrow bandwidth and independence of fluorescent label, providing high stability and accuracy for the encoding method. This synthesis method directly dopes the Raman reporter molecules into an aqueous phase; then, the doped aqueous phase solution is cut into droplets in the oil phase and solidified into beads with the irradiation of ultraviolet light. The preparation process is simple and easy to operate, and the assembly of the encoding materials is not limited by the molecular functional groups. Meanwhile, hydrogel-based REBs with good biocompatibility and low cost can be quickly obtained. Since the method concentrates plenty of Raman reporter molecules inside the microbeads; a high signal-to-noise ratio can be procured without other enhancement methods. We verified the feasibility of applying multiple kinds of Raman reporter molecules and signal intensity ratios of different Raman reporter molecules for preparing the encoded microcarrier. This encoding method solves the limit in encoding number caused by the spectral overlap of similar functional groups, consequently expanding the detection throughput of Raman-encoded SA. Multiplexed

biomolecule detection and concentration response experiments confirmed the good performance of this encoding method with the limit of detection of 52.68 pM. Therefore, the application of reversed-phase suspension polymerization method and ultraviolet light curing for one-step synthesis of Raman-encoded SA has great potential in biomolecule detection.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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