

# Multiplex assays of bladder cancer protein markers with magnetic structural color hydrogel microcarriers based on microfluidics

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## ABSTRACT

Bladder cancer is one of the most common malignant tumors of the urinary system with a significantly increased incidence in recent years. Tumor biomarkers detection with high specificity and sensitivity bears great significance in clinical diagnosis and treatment. Here, we propose a novel microfluidics-based magnetically responsive structural color hydrogel microcarrier as the multiplex detection platform, with the advantages of easy accessibility and high sensitivity. The resin material with the superior mechanical property was applied to prepare the inverse opal as the framework, in whose gaps perfused with a multifunctional hydrogel composed of polyethylene glycol diacrylate (PEGDA), acrylic acid (AA) and Fe<sub>3</sub>O<sub>4</sub> nanoparticles. The composition enabled the microcarrier with high mechanical strength, low non-specific molecular adhesion, and directional magnetic motion ability, which significantly improved the detection efficiency of the platform. The results demonstrated that our detection system had good sensitivity and reliable specificity in the multiplex analysis of bladder cancer-related protein nuclear matrix protein 22, human bladder tumor antigen and fructose-1,6-diphosphate. Furthermore, the microcarriers were proved to have an ideal application prospect in clinical diagnosis and monitoring. Therefore, magnetically responsive structural color hydrogel microcarriers were suitable for the construction of liquid chips for bladder cancer protein markers.

## 1. Introduction

Bladder cancer, one of the most common malignant tumors of the urinary system, is characterized by multicentric onset and high-rate relapse. In recent years, the incidence and mortality of bladder cancer have been on the rise, seriously threatening the survival of patients [1–3]. At present, the application of biomarkers has improved the diagnosis method of bladder tumor, especially protein biomarkers, as the performers of physiological function, featuring high sensitivity and specificity in the early diagnosis and prognosis monitoring of diseases [4]. Multiplex analysis of protein biomarkers is expected more and more urgently in clinical detection. Nowadays, the commonly used methods are to fix protein molecules on the solid phase carriers, such as protein microarray and microplate protein chip technology [5]. While the arrangement positions and fluorescence were used as the coding elements, the quantification of protein molecules was indicated by the

fluorescence intensity of the corresponding antibodies. However, there are still plenty of shortcomings in this work. Firstly, a high steric hindrance effect may exist on the surface of the solid phase carrier, resulting in low efficiency and poor repeatability of biomarkers encoding. And the signal decoding is achieved by special equipment and professional operation. Moreover, the fluorescence of probes is easy to be confused with the encoded fluorescence, which affected the detection accuracy to a large extent [5]. Therefore, a new multiplex analysis platform for bladder cancer biomarkers is still expected to be developed.

Here, we propose a microfluidics-based magnetic structure color hydrogel microcarrier for the quantitative detection of bladder cancer-related protein biomarkers efficiently, as described in the schematic diagram (Fig. 1). Photonic crystal (PhC), based on the long-range ordered nano arrangement, generates photonic band gap effects, and the characteristic reflection peaks can be used as the multiplex encoding elements with the advantages of coding stability and no fluorescent

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interference [6,7]. Particularly, the hydrogel structure, in addition to the similar encoding property, can provide an ideal fixation and reaction platform for the biomarker molecules [8–13]. However, there are still some problems with such microcarriers, for instance, the mobility in the detection process needs to be further improved [14,15], and the effect in the analysis of bladder cancer-related protein biomarkers has not been verified.

In this paper, we have developed a novel structural color hydrogel microsphere as the microcarrier. Ethylene glycol dimethyl Acrylate (EGDMA) was used as the inverse opal support structure, while the pores were filled with a mixture of polyethylene glycol diacrylate (PEGDA), acrylic acid (AA) and  $\text{Fe}_3\text{O}_4$  nanoparticles. PEGDA was used to avoid non-specific binding of protein molecules. AA provided active carboxyl groups for probe ligation and biomolecular detection. Besides,  $\text{Fe}_3\text{O}_4$  nanoparticles could induce the directional motion of the microcarriers under the magnetic field control. Thus, the defect of insufficient braking has been well solved. The results showed that, magnetic structure color hydrogel microspheres had the advantages of low interference, high specificity and efficiency in the multiplex analysis of bladder cancer-related protein biomarkers. Hence, the microcarriers had the ideal detection performance and clinical application potential.

## 2. Experimental section

### 2.1. Materials

Ethylene glycol dimethyl acrylate (EGDMA), polyethylene (ethylene glycol) diacrylate (PEGDA) and 2-hydroxy-2-methylpropiophenone (HMPP) photoinitiator were purchased from Sigma-Aldrich (Shanghai, China). Acrylic acid (AA) was purchased from Alfa Aesar Chemical (Shanghai, China).  $\text{Fe}_3\text{O}_4$  nanoparticles were provided by Nanoeast biotech (Nanjing, China). 2-Morpholinoethanesulfonic acid (MES) was purchased from Amresco LLC (Solon, USA). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and n-hydroxy succinimide (NHS) were purchased from Aladdin (Shanghai, China). Bovine serum albumin (BSA) was purchased from Sigma Chemicals (USA). Human Otoraplin protein, Factor H protein, NuMA protein, anti-human Otoraplin antibody, anti-human Factor H antibody, anti-human NuMA antibody, anti-human Otoraplin antibody-Fluorescein isothiocyanate (FITC), anti-human Factor H antibody-FITC, and anti-human NuMA antibody-FITC were purchased from Abcam (USA). All RNAs-free tips were purchased from Biosharp (China). Silicone oil was purchased from (Shinetsu, Japan). All buffers were prepared with purified water from

Millipore (Bedford, USA). All chemicals were analytical grades or higher and used as prescribed. Urine specimens were taken from the Second Affiliated Hospital of Nanjing Medical University, China. The collection and treatment of clinical specimens were conducted in accordance with the guidelines issued by the ethics committee of the Chinese academy of sciences.

### 2.2. Instruments

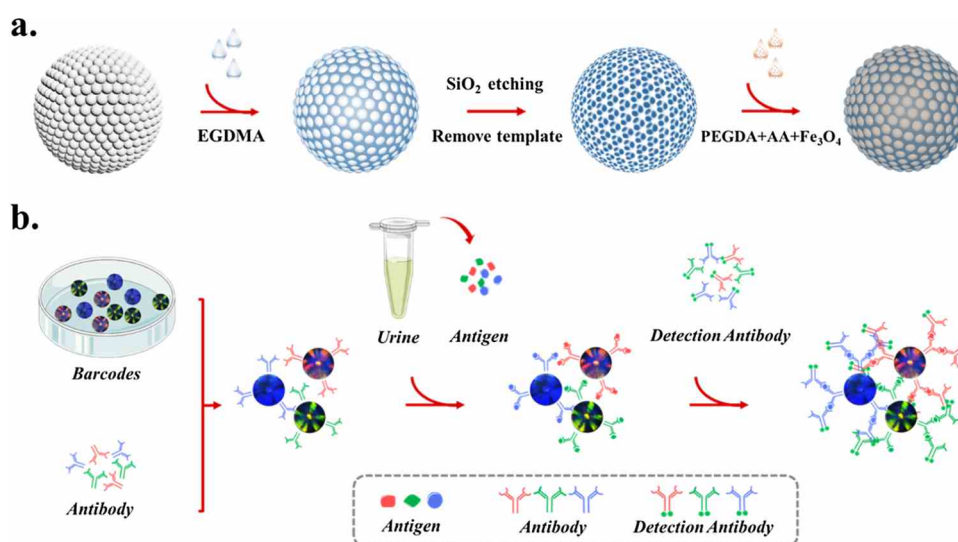
Microfluidics mechanical injection pump and constant pressure pump were provided by Co-microfluidics Technology (Suzhou, China). Each reaction was performed on a thermostatic oscillator (Eppendorf, Germany). The microstructures were characterized by a field emission scanning electron microscopy (SEM, s-300n, Hitachi, Japan). Reflection wavelengths were taken by a metallographic microscope equipped with a fiber optic spectrometer (HR2000, Ocean Optics, USA). The fluorescence intensity of PhC barcodes was detected by a fluorescence microscope (Olympus, CKX41) equipped with the same spectrometer. Multiplex biological analysis images were taken by a stereoscopic microscope equipped with an Olympus camera (Olympus, LG-PS2) and mercury lamp (Olympus, U-RFL-T).

### 2.3. Synthesis of $\text{SiO}_2$ nanoparticles

$\text{SiO}_2$  nanoparticles were synthesized by Stober method. Tetraethyl orthosilicate (TEOS) was added drop by drop to the mixed solution of ethanol (300 mL) and ammonium hydroxide (10 mL). With the hydrogenation of TEOS,  $\text{SiO}_2$  nanoparticles grew continuously with stirring (300 rpm, 30 °C).  $\text{SiO}_2$  nanoparticles were sampled and measured every half an hour to get the desired nanoparticles of different sizes.

### 2.4. Preparation of PhC barcodes

According to the previous work of our research group, PhC barcodes were prepared by microfluidic droplet chip technology [16,17]. The dispersed phase was water suspension of monodisperse silica nanoparticles, and the continuous phase was silicon oil (50 cSt). By means of a microfluidic device, the dispersed phase was cut into droplets and collected in silicone oil (500 cSt). The droplets were evaporated at 75 °C for 12 h, and the internal silica nanoparticles were self-assembled and hardened by heating to form solid microspheres. Then, the silicone oil on the surface of PhC barcodes was cleaned by n-hexane and then transferred to the muffle furnace for calcination at 800 °C for 3 h to



**Fig. 1.** (a) Schematic diagram of the preparation of magnetic structural color hydrogel microcarriers; (b) Schematic diagram of magnetic structural color hydrogel microcarriers for multiple immunoassays.

improve the mechanical strength. To meet the needs of multiplex immunoassay, three kinds of PhC with reflection peaks of 480 nm, 575 nm and 600 nm were applied to prepare magnetic structural color hydrogel microcarriers.

## 2.5. Preparation of magnetic structural color hydrogel microcarriers

The PhC barcodes were used as templates, and EDGMA was utilized to copy the void structure inside the microspheres. As the silica nanoparticles were removed, the inverse opal structure was obtained, which was immersed in a pregel solution contained 20 % PEGDA, 15 % AA, 4 mg/mL  $\text{Fe}_3\text{O}_4$  nanoparticles, and 1% photoinitiator. The pregel were then solidified by UV irradiation for 30 s and used as the microcarriers for multiplex encoding.

## 2.6. Ligation of antibody probe

The antibody molecular probes were ligated to magnetic structural color hydrogel microcarriers by chemical coupling [18]. In MES buffer (pH 6.0), the carboxyl group provided by AA was activated to react with the amino group through the crosslinking effect of EDC and NHS at a constant temperature of 37 °C for 1 h. Then three kinds of microcarriers ligated with different probes were washed with PBS buffer, thus magnetic structural color hydrogel microcarriers coupled with probes for multiplex analysis of protein biomarkers were obtained.

## 2.7. Detection of protein biomarkers

Magnetic structural color hydrogel microcarriers ligated with antibody probes were passivated by PBS containing 1% BSA for 2 h before binding to target protein molecules to avoid non-specific adsorption. After incubation with 10  $\mu\text{L}$  protein markers solution for 1 h, anti-NMP22 antibody-FITC, anti-FDP antibody-FITC, and anti-BTA antibody-FITC at the concentration of 0.1 mg/mL were added to the reaction solution and incubated for 1 h. During the whole process, the reaction solution was oscillated at a constant temperature of 37 °C. The unbound antibody-FITC was then washed off with PBS buffer. Each detection was repeated three times. All the experimental procedures need to be treated in a dark place.

Under the magnetic field, magnetic structural color hydrogel microcarriers were endowed with controllable motion property. In the biological analysis, the directional magnetron effect was used to clean and enrich the microcarriers, which could quickly gather on one side of the reaction tube. Then, a magnetic agitator (300 r/min) was used to increase the contact opportunity and binding efficiency between the microcarriers and the target molecules in the incubation reaction.

In the detection of clinical specimens, urine specimens were collected from the patient through a catheter into the centrifuge tube of 50 mL, and the cells were removed by centrifugation at 2000 r/min for 10 min. Then, the supernatant was taken into a new 50 mL centrifuge tube for use. Magnetic structural color hydrogel microcarriers prepared by PhC barcodes with different reflective wavelengths were used to analyze different protein biomarkers simultaneously. The characteristic reflectance spectra of microcarriers were utilized for encoding and decoding, while fluorescence intensities were used for quantification [19] to realize multiplex analysis of bladder cancer protein markers.

## 3. Results and discussion

### 3.1. Design and characteristics of magnetic structural color hydrogel microcarriers

In a classic experiment, monodisperse silica nanoparticles were assembled into PhC barcodes using a microfluidic droplet device [16–21] (Fig. S1). After solidification and calcination, the silica PhC was used as the templates and soaked in a resin solution of 100 % EGDMA

and 1% photoinitiator, which passed through and filled the gaps between the nanoparticles by capillary force. Then, after UV polymerization for 30 s, the PhC template was corroded by hydrofluoric acid to obtain the EGDMA inverse opal structure. This structure has good stability to be used as a skeleton structure for coding microcarriers. Then, a mixture pregel solution of PEGDA and AA containing  $\text{Fe}_3\text{O}_4$  nanoparticles was prepared for reperfusion, which was perfused into the porous structure and repolymerized under ultraviolet light. After removing the hydrogel on the surface, magnetic structural color hydrogel microcarriers were obtained and collected for multiplex analysis. The microstructure of silica PhC, inverse opal structure and magnetic structural color hydrogel microcarriers was observed by a scanning electron microscope (SEM), respectively. The results showed that the nanoparticles were arranged in a periodic long-range order in PhC barcodes. The hydrogel skeleton replicated from silica colloidal crystal bead (SCCB) templates had the similar highly ordered three-dimensional inverse opal structure and hexagonal arrangement (Fig. 2a–b). Moreover, the magnetic structure color hydrogel microcarrier retained the structural characteristic of periodic order (Fig. 2c). Here, in order to achieve an ideal hydrogel perfusion,  $\text{Fe}_3\text{O}_4$  nanoparticles with the particle size of less than 10 nm were selected (Fig. S2a), and the element spectrum was characterized in Fig. S2b.

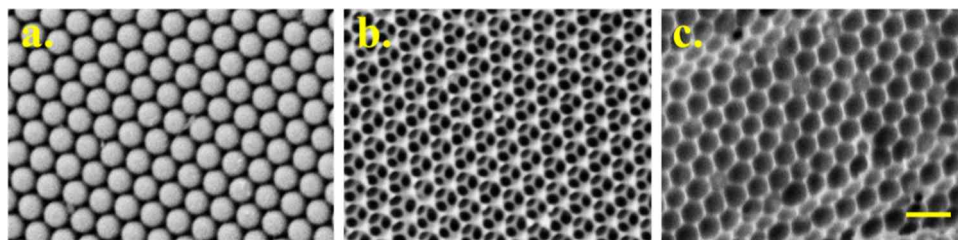
The encoding principle of our platform is based on the characteristic reflection spectra generated from the periodic ordered nanostructure of PhC barcode [22,23]. Similarly, the hydrogel barcodes generate the characteristic reflection peaks and the corresponding structural colors based on the photonic band gap (PBG) effect. Under the normal incident condition, the reflection peak wavelength of the PhC barcode can be estimated by the Bragg equation [24]:

$$\lambda = 1.633dn_{\text{average}},$$

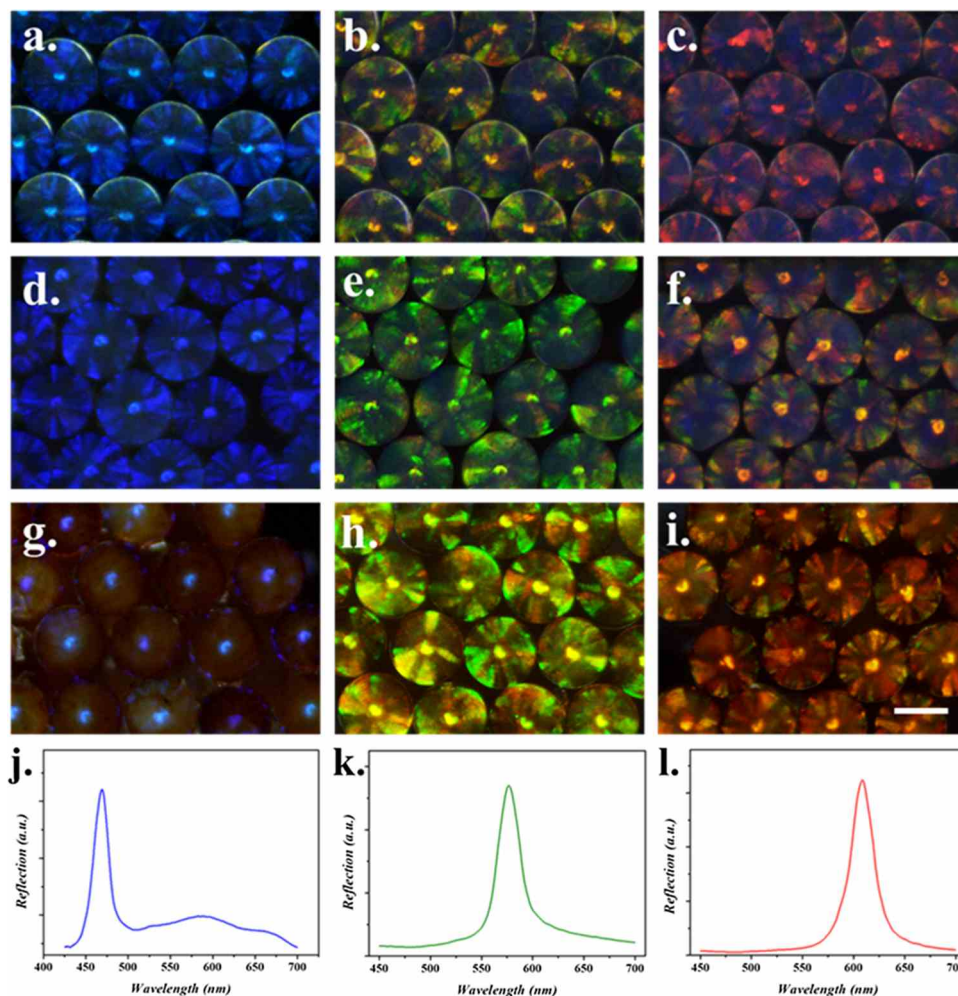
where  $d$  is the distance between the centers of two adjacent nanopores, and  $n_{\text{average}}$  is the average refractive index of PhC barcode. When the composition of the hydrogel and the duty ratio is fixed, the  $n_{\text{average}}$  is constant, and so that the position of reflection peak mainly depends on the diameter of nanopores, which formed by the nanoparticles of the template. By changing the diameter of the nanoparticles, we can obtain a series of PhC barcodes with different reflection spectra and structure colors [25,26].

In the optical images of Fig. 3(a–c), three kinds of  $\text{SiO}_2$  nanoparticles with diameters of 220 nm, 270 nm, and 285 nm were used to prepare PhC barcodes, all of which presented bright structural colors. Magnetic structural color hydrogel microcarriers using photonic crystal microcarrier as the nanostructure template, still exhibit the periodic and orderly nanometer arrangement after the preparation of inverse opal framework and the pre-gel reperfusion, thus possesses the photonic bandgap property. The average refractive index of the medium is calculated according to the materials and components prepared by the microcarrier ( $\text{SiO}_2$  1.54, EGDMA 1.467, PEGDA 1.469, AA 1.442,  $\text{Fe}_3\text{O}_4$  3.0,  $\text{H}_2\text{O}$  1.33) and the spatial composition ratio (the volume of nanoparticles and inverse opal framework accounted for 74 % and 26 %, respectively). The average refractive index of the medium is calculated into the Bragg equation. The calculated wavelengths were 471 nm, 574 nm, and 605 nm, respectively, while the actual spectral reflection peaks measured by the spectrometer were 474 nm, 576 nm, and 608 nm, respectively. The results show that the theoretical calculation values were consistent with the actual measurement value, and the optical properties of this constructed microcarrier accord with the classical law. Based on the periodic ordered microstructure and spherical symmetry, microcarriers can form the same photonic bandgap through the direction of the microsphere center and generate characteristic reflection spectral peaks, showing the corresponding structural color, independent of the angle of detection and observation (Fig. 3(d–f)). According to the Bragg equation, the average refractive index of PhC barcode changes with hydrogel replacing the silica nanoparticles.





**Fig. 2.** (a) SEM image of the surface of PhC barcode; (b) SEM image of the surface of EGDMA inverse opal structure; (c) SEM image of magnetic structural color hydrogel microcarriers. The scale bar is 400 nm.



**Fig. 3.** (a–c) Reflection images of PhC barcodes with three different structure colors; (d–f) Reflection images of the corresponding inverse opal structures; (g–i) Reflection images of magnetic structural color hydrogel microcarriers; The scale bar is 200  $\mu\text{m}$ ; (j–l) Characteristic reflection spectra of magnetic structural color hydrogel microcarriers.

Therefore, the structural colors (Fig. 3(g–i)) and reflective wavelengths (Fig. 3(j–l)) of the magnetic structural color hydrogel microcarriers were different from those of the barcodes but still stable and can be used as the encoding elements for the multiplex analysis of biomarkers [27–30].

### 3.2. Preparation of hydrogels and optimization of conditions

To achieve the mechanical stability of magnetic structural color hydrogel microcarriers, EGDMA was used as the scaffold material of the inverse opal structure. Then, a mixture pregel containing PEGDA, AA and  $\text{Fe}_3\text{O}_4$  nanoparticles was perfused into EGDMA template and polymerized under UV light. PEGDA has the biological characteristic of low

adhesion to proteins so as to eliminate the non-specific binding. AA provides the active carboxyl groups to bind the probe molecules for the coding microcarriers. The addition of  $\text{Fe}_3\text{O}_4$  nanoparticles enabled the microcarriers to conduct controlled motion under the induction of the magnetic field, which improves the detection efficiency. These ingredients endowed the microcarriers with multi-functions and significant advantages in biosensing.

In order to optimize the biological analysis performance of magnetic structural color hydrogel microcarriers, we explored the influence of PEGDA concentration on coding efficiency. Microcarriers with low PEGDA concentration have poor mechanical properties and weak resistance to non-specific adsorption [31,32], while the high

concentration of PEGDA would be too viscous to fill the gaps of the inverse opal microspheres. Here, we synthesized the hydrogel microcarriers based on PhC with the reflection peak of 576 nm, and PEGDA was set to 5 different concentrations to explore their coding effect on target markers. The results showed that hydrogel microcarriers prepared by PEGDA at the concentration of 10 % had the strongest fluorescence intensity for encoding (Fig. 4a). Given the mechanical properties and biomolecular adhesion of the microcarriers, PEGDA with a concentration of 20 % was eventually selected for the preparation of magnetic structural color hydrogel microcarriers.

Similarly, under the condition of constant PEGDA concentration (20 %), we set various concentrations of AA, which was closely related to the ability of hydrogel microcarriers for probe ligation. A high concentration of AA may affect the perfusion efficiency of the mixed pregel into the inverse opal structure. Thus, in the microcarrier preparation system, we chose 15 % AA as the proper concentration to provide active carboxyl groups (Fig. 4b). In addition, the suitable concentration of  $\text{Fe}_3\text{O}_4$  nanoparticles should not only endow the microcarriers with the directional magnetic effect, but also consider the diffusion efficiency of  $\text{Fe}_3\text{O}_4$  nanoparticles in the void of inverse opal structure. Given the concentration-signal effect analysis,  $\text{Fe}_3\text{O}_4$  nanoparticles with a concentration of 4 mg/mL were selected for the subsequent experiments (Fig. S3). In addition, we further explored the magnetic effect of the magnetic structure color hydrogel microcarrier and whose influence on the detection efficiency. Under the magnetic field, magnetic structural color hydrogel microcarriers could realize a rapid directional induction movement and gathered in the direction of the magnetic field (Fig. S4 and Video S1). Moreover, the classical PhC microcarriers and the magnetic structure color hydrogel microcarriers without magnetic field were used as the control group respectively to explore the influence of magnetic control motion on the detection efficiency. The results showed that the signal intensity of the magnetic structural color hydrogel microcarriers was significantly enhanced by the magnetic agitation (Figs. S5 and S6). Due to the microcarriers moving with directivity, there were

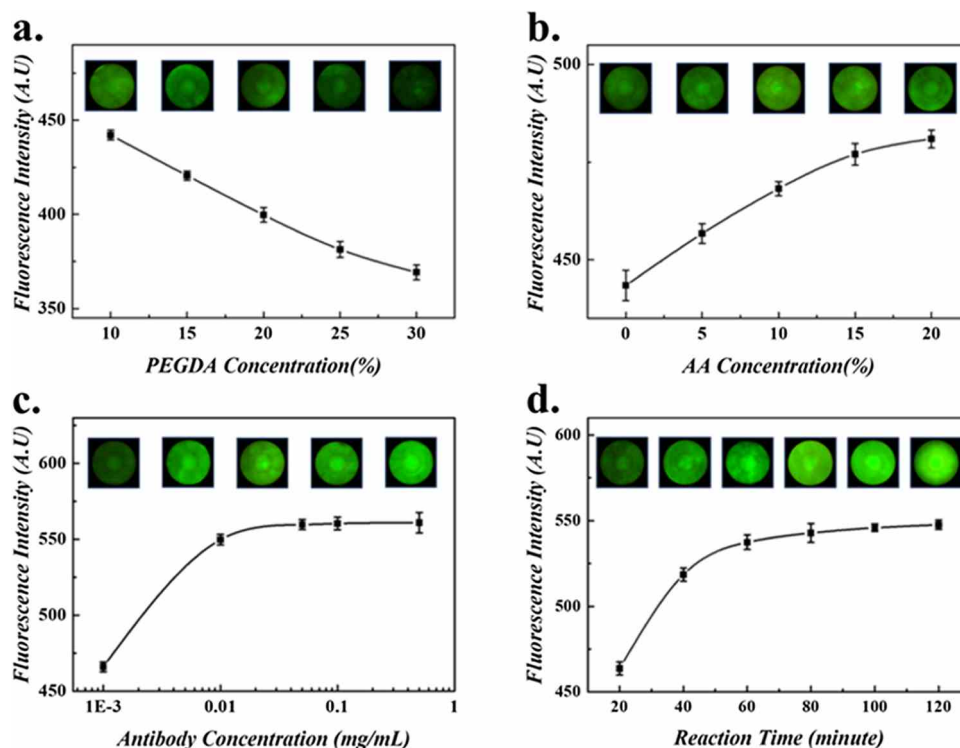
more probabilities for contact with the target markers to combine more fully, thus improving the detection efficiency and sensitivity. Because the lattice structure of the nanocrystalline resin material with the ideal mechanical property was constant, and the binding of targets with microcarrier caused little change of the average refractive index, the structural color change of the microcarriers is not obvious (Fig. S7).

By the covalent chemical reaction between the amino group of the antibody and the activated carboxyl group on the magnetic structural color hydrogel microcarriers, antibodies were modified to the microcarriers as the molecule probes. The dilution concentration of antibodies was optimized on the basis of the above experimental conditions, which were set to five gradients as 0.001 mg/mL, 0.01 mg/mL, 0.05 mg/mL, 0.1 mg/mL and 0.5 mg/mL, respectively. Target biomarkers were identified and quantified by fluorescence intensity analysis. The results indicated that the braking intensity was saturated when antibody concentration reached 0.01 mg/mL (Fig. 4c). Moreover, the fluorescence intensity was stronger as the reaction time of the target biomarkers captured by the antibodies was 1 h (Fig. 4d).

### 3.3. Protein markers detection

Bladder cancer is one of the most common malignant tumors in the urinary system, it is of great clinical significance to a construct multiplex and high-sensitivity detection platform for its early diagnosis and prognosis monitoring [33,34]. Urine specimens have the advantages of easy to collect and non-invasive, and are widely accepted by patients [35–37]. At present, human bladder tumor antigen (BTA) (also called Human Complement Factor H Related Protein, HCFHrp), nuclear matrix protein 22 (NMP22) (also called Nuclear Mitotic Apparatus protein, NuMA), and fibrocyte derived protein (FDP) (also called Otoraplin, OTOR) have been applied clinically as the biomarkers of bladder cancer by the FDA [38–40].

Under the optimized experimental conditions, the specific antibodies of these three biomarkers were used as the probe molecules and binded



**Fig. 4.** (a) The relationship between the of fluorescence intensity and the concentration of PEGDA; (b) The relationship between fluorescence intensity and the concentration of AA; (c) The relationship between fluorescence intensity and the concentration of antibody; (d) The relationship between fluorescence intensity and the reaction time.

to the surface of the magnetic structural color microcarriers. The multiple biomarkers in the samples were detected by BSA blocking strategy to avoid the adsorption of non-specific protein molecules (Fig. S6). Biomarkers with the concentrations of 1 ng/mL ~ 1 mg/mL were quantitatively added into the detection system to analyze the measurement range and detection limit. The concentration-effect curves of BTA, NMP22 and FDP were respectively shown in Fig. 5(a–c). The results showed that as the concentration increasing from 1 ng/mL to 1 mg/mL, the fluorescence intensity grew correspondingly with the correlation coefficients all over 0.990. The fitting equation made according to the detection results were as followed (x-axis, the concentration of protein biomarkers; y-axis, the fluorescence intensity of magnetic structural color hydrogel microcarrier):

$$y = 135.02x^2 + 462.83x + 423.92 \quad (R^2 = 0.9970) \text{ for BTA};$$

$$y = 94.53x^2 + 382.77x + 432.82 \quad (R^2 = 0.9925) \text{ for NMP22};$$

$$y = 156.89x^2 + 545.71x + 494.56 \quad (R^2 = 0.9912) \text{ for FDP};$$

Furthermore, in the multiplex immunoassay of protein markers, it is necessary to mention that the reliability of cross-reaction, which is crucial to the detection specificity of the multiplex analysis platform. In our system, three protein biomarkers were detected in a same reaction tube. The cross-reactions were evaluated by analyzing the fluorescence intensity of each specific analyte (at a concentration of 10  $\mu$ g/mL). When the concentration of the other two protein biomarkers increased from 0  $\mu$ g/mL to 100  $\mu$ g/mL in the solution, the fluorescence intensity of the specific analyte changed no more than 5%. This result indicated that the cross-reaction of multiple protein biomarkers did not affect the specificity of this multiplex detection platform (Fig. 5d). Under the same experimental conditions, we evaluated the encoding spectral peaks and fluorescence intensity of the magnetic structural color hydrogel microcarriers, and the results showed that the platform had an ideal repeat-ability (Table S1).

### 3.4. Clinical application of magnetic structural color hydrogel microcarriers

In this platform, three kinds of SCCBs with characteristic reflection peaks of 480 nm (blue), 575 nm (green), and 600 nm (red) were used as the templates for preparing magnetic structural color hydrogel microcarriers, respectively. Then the three types of microcarriers were modified with different antibody probes to explore their multiplex quantitative analysis capability of corresponding bladder cancer protein biomarkers in the reaction solution. The antigen-antibody binding efficiency was quantified by fluorescence intensity of FITC. The results showed that the magnetically responsive structural color hydrogel microcarriers bound with the target biomarkers specifically (Fig. 6a (i-iii and v-vii) and Fig. 6b). Additionally, the structural colors of PhC were employed as barcodes to distinguish the microcarriers modified with probes so that they could detect multiple biomarkers simultaneously (Fig. 6a (iv and viii) and b).

To demonstrate the application potential and the reliability of the magnetic responsive structural color hydrogel microcarriers for multiplex assay in clinical diagnosis, we used the microcarriers to detect urine protein biomarkers in bladder cancer patients (BC group), non-bladder cancer patients (non-BC group), and healthy controls (NC group). Clinical specimens were collected from the Second Affiliated Hospital of Nanjing Medical University in China using standard urine collection technique. Fluorescence intensity of urine specimens was detected in patient groups and healthy control groups. Taking pathological diagnosis as the gold standard for bladder cancer, the receiver operating characteristic (ROC) curve under 95 % confidence interval was analyzed by our detection platform to evaluate the efficiency. As shown in Fig. 7 (a–f), the BC group exhibited the highest fluorescent intensity, indicating that our method had reliable specificity and ideal application potential. In addition, it was worth mentioning that this multiplex analysis platform required a lower detection volume of 10  $\mu$ L and without complex protein extraction. Therefore, multiplex immunoassay of trace protein biomarkers in small sample volumes could be achieved by magnetic structural color hydrogel microcarriers.

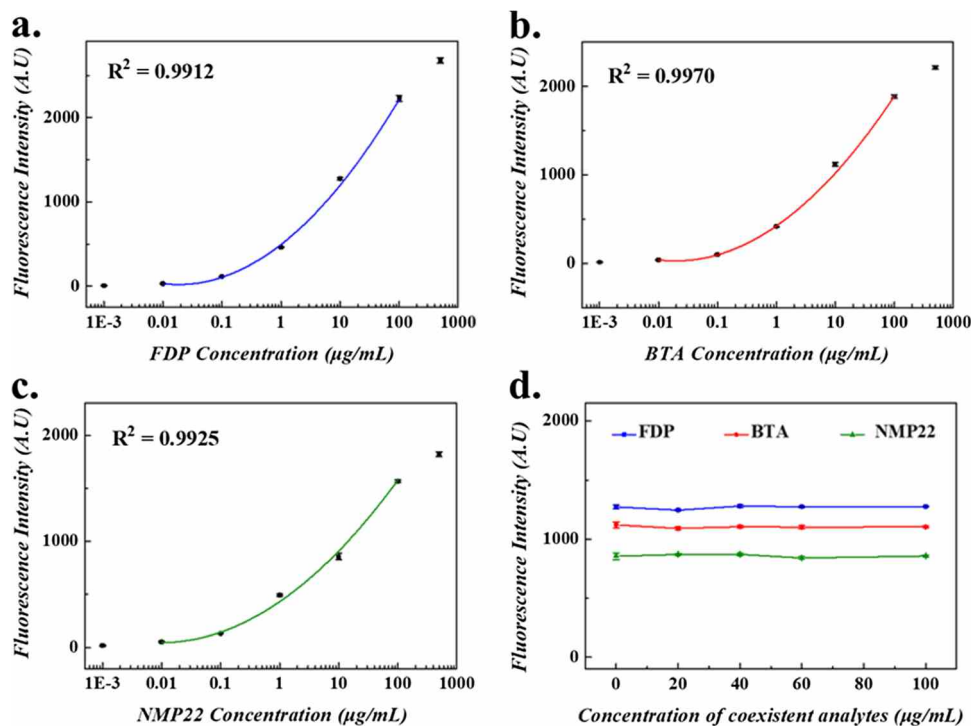
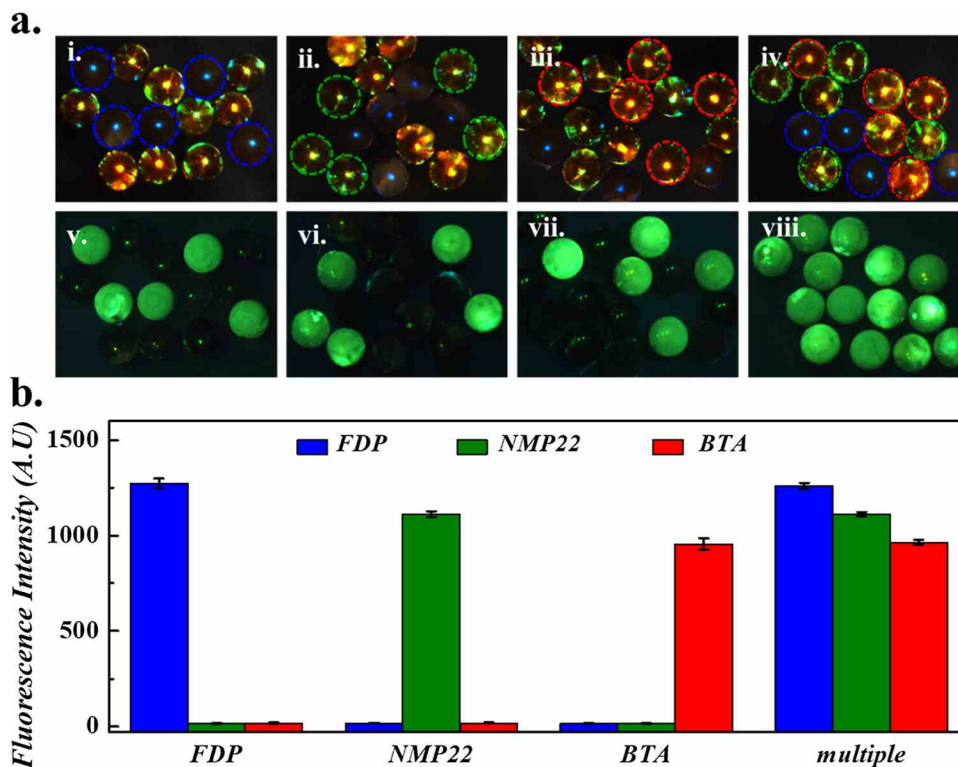
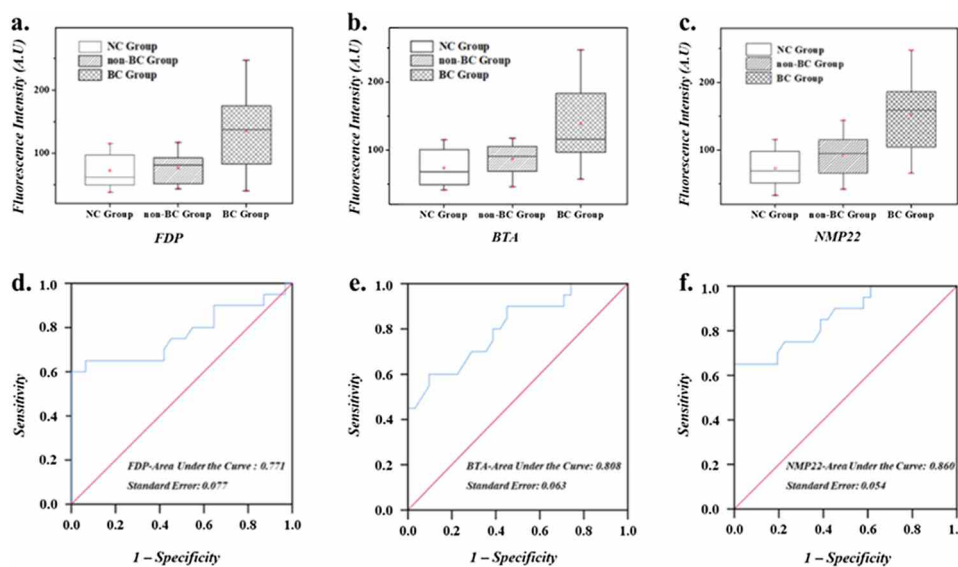


Fig. 5. (a–c) Correlation curves between the concentration of biomarkers in the range of 1 ng/mL ~ 1 mg/mL and fluorescence intensity; (d) Cross-reaction curves of biomarkers at the concentration of 10  $\mu$ g/mL.





**Fig. 6.** (a) Images of optical microscope (i–iv) and fluorescence (v–viii) of magnetic structural color hydrogel microcarriers after multiplex biological reactions; (b) Fluorescence intensity analysis of barcodes after multiplex biological reactions.



**Fig. 7.** Boxplot and ROC curves of multiplex detection of bladder cancer protein biomarkers by magnetic structural color hydrogel microcarriers. (a, d) BTA analysis of clinical specimens; (b, e) FDP analysis of clinical specimens; (c, f) NMP22 analysis of clinical specimens.

#### 4. Conclusion

In summary, we developed a novel magnetically responsive structural color hydrogel microcarriers based on microfluidic for multiplex detection of bladder cancer protein markers. The microcarriers utilized EGDMA resin as the stable skeleton of inverse opal structure and then were perfused with multifunctional hydrogel. When used for the assay of bladder cancer protein markers BTA, NMP22, and FDP, they exhibited excellent properties such as preventing non-specific binding, ligating biological probes, and high detection efficiency. It was showed that the

detection volume of this platform was as low as 10  $\mu$ L, while the concentration-effect curves ranged from 10 ng/mL to 0.1 mg/mL. In addition, the analysis showed that the detection limits of BTA, NMP22, and FDP were 153.6 ng/mL, 329.1 ng/mL, and 258.8 ng/mL, respectively. Furthermore, the results of immunological cross-reaction showed a reliable specificity. They also exhibited a desired detection performance in the detection of clinical specimens. Therefore, magnetic structural color hydrogel microcarriers were promising in multiplex detection of bladder cancer protein markers.

## CRediT authorship contribution statement

Yefei Zhu and Huan Wang conceived the idea and designed the experiment. Xiaowei Wei, Hui Zhang and Huan Wang conducted experiments and data analysis. Xiaowei Wei, Feika Bian and Huan Wang wrote the manuscript.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.snb.2021.130464>.

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