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Fabrication of Immune-Affinity Monolithic Array for Detection of α-Fetoprotein and Carcinoembryonic Antigen

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***Abstract*—**In this paper, we presented a highly sensitive immune-affinity monolithic array for detection of α-fetoprotein (AFP) and carcinoembryonic antigen (CEA). Firstly, the epoxy functionalized monolith arrays were fabricated using UV initiated copolymerization method. Scanning electron microscopy (SEM) image showed that the poly(BABEA-*co*-GMA) monolith exhibited a well-controlled skeletal and well-distributed porous structure. Then, AFP and CEA immune-affinity monolithic arrays were prepared by immobilization of AFP and CEA antibodies on epoxy functionalized monolith arrays. With a non-competitive immune response format, the presented AFP and CEA immune-affinity arrays were demonstrated as an inexpensive, flexible, homogeneous and stable array for detection of AFP and CEA.

***Keywords*—**Chemiluminescent detection, immune-affinity, monolithic copolymer array, UV-initiated copolymerization.

I. Introduction

ENZYME-linked immune-sorbent assay (ELISA) is widely used in medical diagnosis and biochemical analyses to detect proteins based on their binding to the immobilized antibodies. A primary antibody immobilized on a solid surface is used to capture the antigen, and a secondary labeled antibody is used to detect the captured antigen. The binding of the secondary antibody is quantitated by measuring the activity of an enzyme bound to the secondary antibody. Most of ELISAs today are performed in 96-well plates. Dedicated instruments have been developed to automate the assay, including robotic pipetters, plate washers, and optical colorimetric detectors. However, the process of ELISA is slow (e.g. several hours), and requires large amount of samples and reagents. Miniaturized chemical systems on immunoassay are garnering great interest [1]-[3]. Miniaturized immunoassays, nevertheless, place greater demands on handling accuracy; and attempt to address this with more precise machinery naturally translate to higher cost. This has led to development of an alternative approach to handle small liquid volumes without complex or precise machinery needed [4], [5].

Polymeric monoliths have already developed into important materials in the areas of separation science [6]. Monoliths consist of a single continuous network of porous materials that provide high surface area, tunable functionality, easy fabrication, and low cost. Due to these advantages, polymeric monoliths can be seen as excellent supports for the immune-affinity materials. Two methods that are commonly used to prepare monoliths are thermal polymerization [7] and UV-initiated polymerization [8]. UV-initiated polymerization is more desirable for fabrication of monolithic arrays, because it can provide spatial resolution with a photo-mask.

Bisphenol A based epoxy acrylate (BABEA) is one of the most widely used commercially available UV curable oligomer, because of its fast curing speed, low-cost, good pigment wetting, high gloss, hardness, and chemical resistance of the cured films [9], [10]. In this paper, BABEA is introduced as a cross-linker for facile manufacturing of epoxy-functional monolith arrays through on-suit UV copolymerization using GMA as the functional monomer and PEG 200 as the porogen. A fabrication procedure is presented to fabricate epoxy-functionalized monolith arrays. Firstly, the poly (BABEA-*co*-GMA) monoliths exhibit a well-controlled skeletal and porous structure, which provide high surface area and increase binding amount of biological molecules. Secondly, the complementary photo-mask is used, so the poly (BABEA-*co*-GMA) monolith is polymerized at the test zone.

AFP, an oncofetal glycoprotein with a molecular weight of approximately 70,000 Da, is well known as a tumor marker. The concentration of AFP in healthy adults is typically below of 25 ng/mL. Increased serum AFP levels have been considered as early indication of some cancerous diseases including hepatocellular cancer, yolk sac cancer, liver cancer and nasopharyngeal cancer. Thus, it is very important to explore a rapid detection method for AFP. CEA, a glycoprotein normally found in the tissue of developing baby in the womb. The blood level of this protein disappears or becomes very low after birth. In adults, abnormal levels of CEA may be seen as a sign of lung or breast cancer. Here, epoxy groups located within porous monoliths permit binding with AFP and CEA antibodies through an epoxy-amino group reaction, which is applied for construction of AFP and CEA immune-affinity monolithic arrays [11], [12].

II. Materials and Methods

A. Reagents and Materials

BABEA photo-resist (EBECRYL 600) was purchased from UCB (Belgium). 1-Hydroxy cyclohexyl phenyl ketone

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(Irgacure 184) from Ciba Specialty Chemicals (Switzerland) was used as a photo-initiator. Human AFP, Human CEA, AFP antibody, CEA antibody, HRP-conjugated AFP antibodies, and HRP-conjugated CEA antibodies were purchased from Shuangliuzhenglong Chemical and Biological Articles Co. Ltd. (China). Bovine serum albumin (BSA) was purchased from Sigma (USA) and prepared in 0.1 mol/L phosphate buffer solution (PBS, pH=7.0). Luminol and p-Iodophenol (PIP) were purchased from Nanjing Searchbio Co. Ltd. (China). Dimethylsulfoxide (DMSO) and GMA were purchased from Aladdin-Reagent (Shanghai, China). Other chemicals were of analytical-reagent grade. Water was purified with a Milli-Q Advantage A10 (USA). A series of photo-masks with different design were purchased from the Fifty-fifth Research Institute of China Electronic Science & Technology Group Company (China).

B. Instruments

A UV curing chamber equipped with a 365-nm UV light source was obtained from Zhongtian Coating (Baoding, China). Chemiluminescence (CL) detection was performed on a MPI-A Luminescence Analyzer (Ruimai Analytical Instrument, Xi'an, China). SEM analyses were performed on a Hitachi FE-SEM S-4800 (Tokyo, Japan).

C. Preparation of Stock Solutions and Pre-Treatment of Glass Substrates

The pre-polymer solution was prepared by mixing the cross-linker BABEA, functional monomer GMA, and photo-initiator Irgacure 184, and porogen PEG 200. The volume ratio of porogen PEG 200 to cross-linker BABEA and the copolymerization time were investigated and optimized respectively. The composition of the pre-polymer solutions used for optimizing the volume ratio of PEG 200/BABEA was shown in Table I.

A luminol stock solution (0.01 M) was prepared by dissolving 177 mg of luminol (Nanjing Searchbio, China) in 100 ml of 0.1 mol/l NaOH (0.1 M) and was kept in dark. A p-Iodophenol (PIP) stock solution (0.01 M) was prepared by dissolving 110 mg of PIP (Nanjing Searchbio, China) in 5 mL of DMSO and then dilution with water to 50 mL, and was kept in dark. CL substrates were freshly prepared by adding 50 µL of luminol stock solution, 50 µL of PIP stock solution, and 3.4 µL of H_2O_2 (3% v/v) to 900 µL of phosphate buffer (0.2 M, pH 8.5).

Glass substrates were pre-treated with a vinyl silanizing agent that anchored the monolith onto the surface. After treatment with 0.1 M NaOH and 0.1 M HCl for 1 h each and rinsing with water until neutralization (pH 7.0), the glass slides were dried on a heating plate at 60°C for 30 min. Subsequently, the glass slides were immersed in a 1:9 (v/v) mixture of γ-MAPS and methanol at 60 °C for 12 h. Finally, the glass slides were rinsed with methanol and water to flush out residual reagents.

D. Fabrication and Characterization of Immune-Affinity Monolithic Array

The fabrication procedure of immune-affinity array included two steps: fabrication of monolithic array and immobilization of AFP or CEA antibodies (Fig. 1). The monolithic arrays were fabricated using UV-initiated copolymerization. Briefly, the pre-polymer solution was firstly coated onto the pre-treated glass substrate at the speed of 3000 rpm for 20 seconds. Subsequently, a photo-mask of designed pattern printed on a transparency sheet was attached onto the top substrate surface. After that, UV exposure was performed with 300 mJ/cm^2 dose at wavelength of 365 nm for several seconds. Clear areas of the photo-mask allowed transmission of UV light and curing the mixture on illuminated sites, while dark areas of the mask blocked UV light and left the covered regions un-polymerized solution. Then, un-polymerized mixture was removed by washing with the acetonitrile-water solution (50:50, v/v). Finally, the monolithic arrays were dried under vacuum. The immune-affinity monolith arrays were fabricated through an epoxy-amino group reaction. Firstly, 10 µL of AFP or CEA antibodies (0.1 mg/mL) was added onto designed spot and incubated for 12 h at 20 °C. After washing three times with 0.1 M PBS (pH 7.0), the AFP or CEA immune-affinity monolith arrays were blocked with 5 µL of 1% BSA for 2 h. Finally, the immune-affinity monolith arrays were washed with 0.1M PBS (pH 7.0) and stored in 0.1 M PBS (pH 7.0) at 4 °C when not in use.

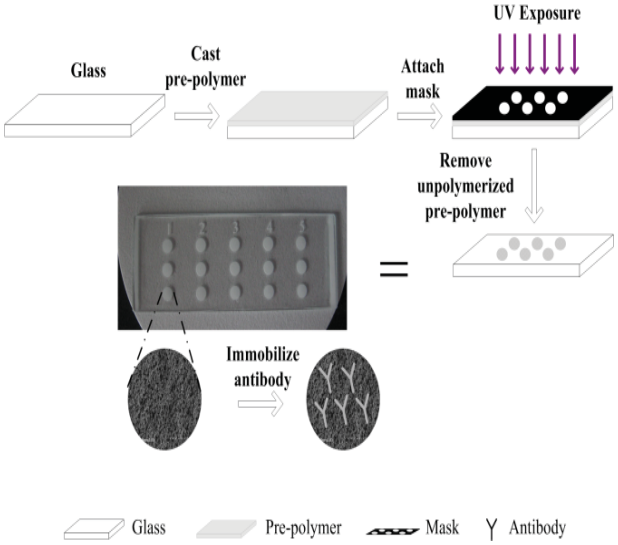

Fig. 1 Scheme of fabrication process of immune-affinity monolith array

The characterization of immune-affinity monolith array was based on non-competitive immune response method (Fig. 2). First, 10 µL of AFP or CEA standard solution was added to immune-affinity monolithic spot. After pre-incubation for 30 min at room temperature, the monolithic spot was washed three times with 3×5 µL 0.1 M PBS (pH 7.0), containing 0.05% Tween-20 (PBST). Then, 10 µL of HRP-conjugated AFP or

CEA antibody were added to the spot. After pre-incubation for 30 min at room temperature, the immune-affinity monolith spot was washed again three times with 3×5 μL 0.1 M PBS (pH 7.0), containing 0.05% Tween-20 (PBST). Finally, the immune-affinity monolith array was placed on the photomultiplier (PMT), and 10 μL of CL substrate was added to the monolithic spot. The CL signals were captured and recorded by the detector.

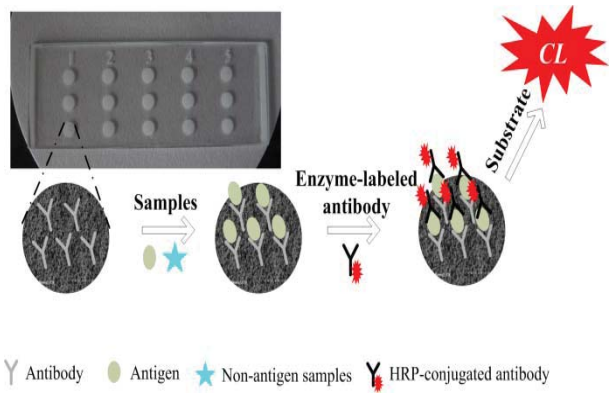

Fig. 2 Scheme of the non-competitive immune response method for detection of AFP or CEA

III. Results and Discussion

A. Optimization of Fabrication Conditions

The volume ratio of porogen PEG 200 to cross-linker BABEA is a key factor for the properties of polymeric monoliths prepared by the presented method. In our experiments, PEG 200 was selected to improve the homogeneity and penetrability of monolithic matrix. If the volume ratio of PEG 200/BABEA is too low or too high, the internal structure of monolith would be unsatisfied. We evaluated the volume ratio of PEG 200/BABEA by SEM image.

TABLE I
Composition of the Pre-Polymer Solution Used for Preparation of Monolithic Copolymer Arrays

	A	*B*	*C*	*D*
PEG 200 (mL)	0	20	50	80
GMA (mL)	3	3	3	3
BABEA (mL)	10	10	10	10
Irgacure 184 (mg)	1	1	1	1
UV exposure time (s)	30	30	30	30

The UV-induced copolymerization time was fixed at 30s. Fig. 3 (A) shows the lucent monolith A has a dense structure. With PEG 200 added, a nano-porous structure was obtained in the translucent monolith B (Fig. 3 (B)). And a well-controlled skeletal and well-distributed porous structure was obtained in the monolith C (Fig. 3 (C)). With more PEG 200 added, the porosity of monolith D was improved (Fig. 3 (D)), but the polymer rigidity became poor. The best volume ratio of PEG 200 to BABEA was found to be 5:1, which provided a well-controlled skeletal and well-distributed porous inner-structure, and has good polymeric rigidity for following experiments.

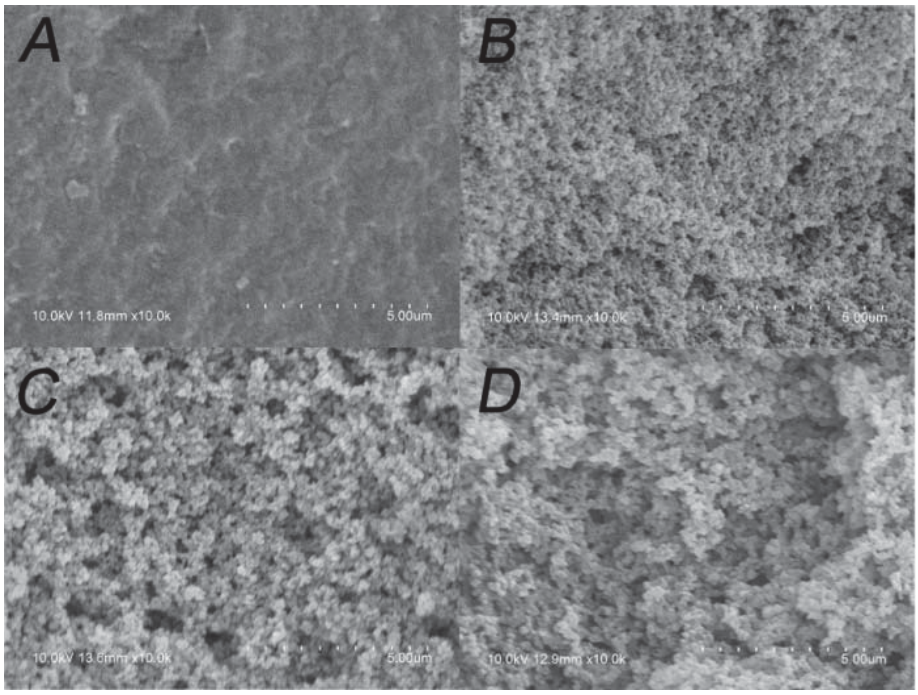

Fig. 3 SEM images of internal structures of monolith (A), (B), (C), and (D)

The UV-induced copolymerization time is another key factor for fabrication of copolymer. We evaluated the UV-induced copolymerization time by measuring the Monomer Content (gram per gram), which was calculated according to:

$$W = \frac{W_0 - W_1}{W_0} \quad (1)$$

where, W is the Monomer Content, W_0 is the weight of dried copolymers, and W_1 is the weight of dried copolymer after extraction in acetonitrile. All experiments were carried out in triplicate, and the average values were reported.

The UV-induced copolymerization time was 15, 20, 25, 30, 35, and 40 seconds, respectively. Fig. 4 showed that the Monomer Content of poly (BABEA-*co*-GMA) as well as the values at different copolymerization time. As the copolymerization time increased from 15s to 30s, the Monomer Content showed greatly decreased from 6.82% (15s) to 3.81% (30s). After the copolymerization times exceeded 30s, the Monomer Content changed litter due to the functional monomer (GMA) total reacted with BABEA. In consideration of energy consumption, this paper used 30 s as the UV-induced copolymerization time.

B. Uniformity of Immune-Affinity Monolith Array

The uniformity and stability of the immune-affinity monolith array was assessed by intra-assay and inter-assay coefficients of variation (CV). The intra-assay CV was the relative difference between five determinations of one sample (0.1 ng/mL of AFP or CEA standard solution) on the same immune-affinity monolith array. The inter-assay CV was the difference between the measurements of the same sample (0.1 ng/mL of AFP or CEA standard solution) on five different immune-affinity monolithic copolymer arrays. As shown in Table II, the intra-assay CVs and inter-assay CVs of AFP immune-affinity monolith arrays were measured to be 3.95%, and 8.52%, respectively. The intra-assay CVs and inter-assay CVs of CEA immune-affinity monolith arrays were measured to be 4.11%, and 8.16%, respectively. The low value of intra-assay CV indicates that each spot on the immune-affinity monolith array was homogeneous. Sometimes, the sticky pre-polymer solution could not be spin-coated uniformly, which affected the performance of immune-affinity monolith array. Considering this factor, the inter-assay CV of 8.52% (AFP immune-affinity array) and 8.16% (CEA immune-affinity array) were acceptable. The accepted inter-assay CV indicates that the presented fabrication method for preparation of immune-affinity arrays was stable.

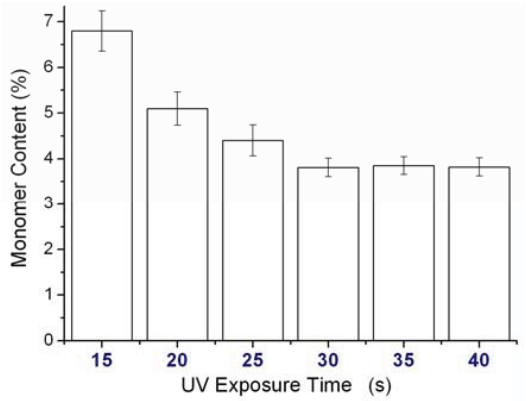

Fig. 4 The Monomer Content of the poly (BABEA-*co*-GMA) with different UV exposure time

TABLE II
INTRA-ASSAY CVS AND INTER-ASSAY CVS OF IMMUNE-AFFINITY ARRAYS

	AFP immune-affinity array	CEA immune-affinity array
Intra-assay CVs (%)	3.95%	4.11%
Inter-assay CVs (%)	8.52%	8.16%

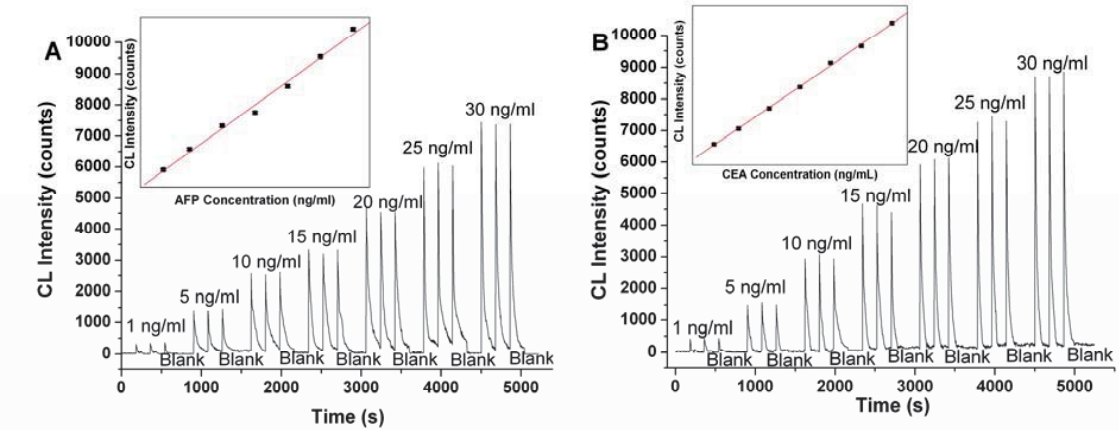

Fig. 5 Dose-response curves (A) AFP immune-affinity array, (B) CEA immune-affinity array: The dose-response curves of AFP and CEA showed a linear range from 0 to 30ng/mL with a correlation coefficient of 0.99. The regression equations were I=81.3+236.7C, (AFP, R^2=0.99), and I=97.3+290.1C (CEA, R^2=0.99), respectively, where I is the CL intensity, and C is the concentration of standard solution.

C. Performance of Immune-Affinity Monolith Array

The performance of the immune-affinity monolith arrays was demonstrated in terms of CL detection of AFP or CEA standard solution with different concentrations. The CL intensities from immune-affinity arrays for AFP and CEA increased with the increasing concentrations of analytes (Fig. 5). The dose-response curves of AFP and CEA showed a linear range from 1.0 to 30 ng/mL with a correlation coefficient of 0.99. The regression equations were I=81.3+236.7C, (AFP, R^2=0.99), and I=97.3+290.1C (CEA, R^2=0.99), respectively, where I is the CL intensity, and C is the concentration of standard solution.

The limit of detections for AFP immune-affinity monolith array was 0.68 ng/mL at a signal to noise ratio of 3, which meets well the requirement for AFP early clinical diagnosis (threshold for positive: 25 ng/mL). The limit of detections for CEA immune-affinity monolith array was 0.67 ng/mL at a signal to noise ratio of 3, which meets well the requirement for CEA early clinical diagnosis.

IV. Conclusion

In this paper, a simple fabrication procedure is presented to fabricate epoxy-functionalized monolith arrays. BABEA is introduced as cross-linker for the facile manufacturing of porous epoxy-functionalized monolith array through photo-patterned UV-initiated copolymerization using glycidyl methacrylate (GMA) as the functional monomer and PEG 200 as the porogen. The method provides poly (BABEA-*co*-GMA) monolith with several excellent properties, including fast-curing, high replication, well-distributed porous structure, and existence of epoxy groups on the copolymer surface for later immobilization of proteins. These favorable features make the poly (BABEA-*co*-GMA) monolith as a promising porous material for fabrication of immune-affinity array. Although these advantageous features were exemplified with the fabrication of AFP and CEA immune-affinity monolith arrays, the monolith arrays are applicable also for other protocols. Also, the fabrication procedure can be applied to manufacture other immune-affinity array such as mycotoxins immune-affinity array, or other tumor markers. These results demonstrate that the monolithic poly (BABEA-*co*-GMA) arrays can provide a sensitive, low-cost, and reliable tool for detecting various biomarkers in biological specimens.

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A new soft lithographic route for the facile fabrication of hydrophilic sandwich microchips (Electrophresis, 2012)

New method for fabricating an α-fetoprotein affinity monolithic polymer array (Journal of Applied Polymer Science, 2015)

UV-initiated copolymerization route for facile fabrication of epoxy-functionalized micro-zone plates (Journal of Applied Polymer Science, 2014)

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