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Fabrication of protein microarrays for alpha fetoprotein detection by using a rapid photo-immobilization process

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ABSTRACT

In this study, protein microarrays based on sandwich immunoassays are generated to quantify the amount of alpha fetoprotein (AFP) in blood serum. For chip generation a mixture of capture antibody and a photoactive co-polymer consisting of N,N-dimethylacrylamide (DMAA), methacryloyloxy benzophenone (MaBP), and Na-4-styrenesulfonate (SSNa) was spotted onto unmodified polymethyl methacrylate (PMMA) substrates. Subsequently to printing of the microarray, the polymer and protein were photochemically cross-linked and the forming, biofunctionalized hydrogels simultaneously bound to the chip surface by short UV- irradiation. The obtained biochip was incubated with AFP antigen, followed by biotinylated AFP antibody and streptavidin-Cy5 and the fluorescence signal read-out. The developed microarray biochip covers the range of AFP in serum samples such as maternal serum in the range of 5 and 100 ng/ml. The chip production process is based on a fast and simple immobilization process, which can be applied to conventional plastic surfaces. Therefore, this protein microarray production process is a promising method to fabricate biochips for AFP screening processes.

1. Introduction

Alpha fetoprotein (AFP), a glycoprotein with a molecular weight of approximately 70 kDa, is the most abundant plasma protein found in human fetus. However, the plasma level decreases rapidly to normal adult levels during the first year of life. Normally, AFP is present only at rather low levels in the blood of healthy people (reference value <10 ng/ml) [1]. Certain types of cancers and liver diseases are known to lead to increased AFP levels. Accordingly, AFP is widely used as a serum biomarker for hepatocellular carcinoma (HCC) screening in patients [2] and for HCC diagnosis [3]. Unusual AFP levels during pregnancy in the maternal blood or in the amniotic fluid are taken as serological soft markers for congenital malformation (e.g. open neural tube defects, anencephaly) or chromosome anomalies. An unusually low value (depending on the stage of gestation) might serve as an indication of the presence of the Down-Syndrome (Trisomie-21) in the developing fetus [4–7].

As of today several techniques have been developed for AFP detection, for example electrochemical immunosensors [8,9], colorimetric detection [10–12] and piezoelectric biosensors [13]. Most of these techniques utilize covalent binding reactions to immobilize the antibody onto the sensing surface [8–11]. Immobilization via covalent binding requires several steps including surface preparation and activation to provide appropriate reactive surface groups, and antibody immobilization. In general, immobilization by practically all methods know today is a multi-step process which is time and resource consuming and increases the risk of quality problems in the production process.

The surface area available for binding of the probe molecules is an important factor for immobilization. When self assembled monolayers are employed, the surface density of capture molecules is intrinsically limited. Additionally, at high surface concentrations the intermolecular distances of the bound molecules are decreased, which might influence the accessibility for target molecules. A too dense binding can result in steric hindrance and might reduce the efficiency of immobilization process [14,15]. To increase the surface density of accessible biomolecules on a sensor surface, immobilization based on surface-attached very thin hydrogel pads has been developed which allow a more three dimensional arrangement of the molecules [15,16].

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In the study described here, the protein immobilization is performed by a rapid photoreaction of a photopolymer with an unmodified PMMA substrate. The photoactive polymer is a terpolymer based on water soluble dimethylacrylamide (DMAA), the photo-crosslinker methacryloyloxy benzophenone (MaBP) and Na-4-styrenesulfonate (SSNa). A mixture of the photopolymer and the protein capture molecules is printed onto the substrate, followed by brief UV-irradiation. The described photo-immobilization process is applied to fabricate sensor chips for AFP detection. The detection range, sensitivity and specificity of this protein microarray biochip are determined.

2. Materials and methods

2.1. Materials

Commercial grade PMMA slides, which were used as substrates, were cleaned with methanol for 5 min before printing. Capture antibody (mouse anti-human AFP) and detection antibody (biotinylated chicken anti-human AFP) were obtained from R&D Systems (UK). AFP antigen was obtained from MyBiosource (USA). Streptavidin-Cy5 was purchased from Life Technologies (USA). Bovine serum albumin (BSA) used as a blocking was obtained from Sigma Aldrich (A7030). Two cytokines were used for the specificity tests, which are tumor necrosis factor alpha (TNFα) and interleukin (IL-8) purchased from Gibco (Life Technology). 10 mM phosphate buffered saline pH 7.4 (PBS; Sigma Aldrich, St. Louis, MO) was used to dilute the capture antibody and streptavidin-Cy5. A dilution buffer (DY995, R&D Systems, UK) was used to dilute detection antibody and AFP antigen.

2.2. Microarray fabrication

The printing solution is prepared from a mixture of the proteins capture molecules and a photoactive copolymer consisting of N,N-dimethylacrylamide (DMAA, 92.5 mol% (w/w)), methacryloyloxy benzophenone (MaBP, 5 mol% (w/w)), and Na-4-styrenesulfonate (SSNa, 2.5 mol% (w/w)) (Fig. 1). The copolymer was synthesized using a standard free radical polymerization process as described previously [17]. The copolymer was dissolved in deionized (DI) water at a concentration of 10 mg/ml prior to mixing with protein solutions which were diluted in PBS to yield the concentrations given in the individual experiments. The final polymer content in printing solution was 1 mg/ml. Printing of the protein arrays was performed under clean room conditions at a temperature of 22 °C and a relative humidity of 40%. The volume per spot was 1.6 nl. Untreated commercial PMMA slides were used as substrates. Protein immobilization is achieved through irradiation with UV-light at a wavelength of 254 nm with energy of 0.5 J/cm^2. A schematic of the layout of the AFP protein microarray is shown in Fig. 2.

2.3. Assay procedure

For isolating reaction sites on larger substrates carrying multiple microarrays, 25 μl Frame Seals (Thermo scientific) were used to create individual wells. The surfaces of the protein microarrays were blocked with 0.1% (w/v) BSA in PBS for 1 h to reduce non-specific binding. After that, the microarrays were incubated with various concentrations of AFP antigen for 2 h, 2 μg/ml of detection antibody for 2 h, and streptavidin-Cy5 in a ratio of 1:200 for 15 min, respectively. Before every step of incubation, protein microarrays were washed with PBS to remove unbound molecules from the biochip. The fluorescence intensity was read out after completing the assay.

In order to avoid probes with non-specific binding of blood components to the surfaces, all test liquids were diluted 1:100 using the dilution buffer given above. The thus obtained AFP standard solutions had the following concentrations: 0.000, 0.005, 0.010, 0.025, 0.050, 0.100, 0.250, 0.500, 1.000, 2.500, 5.000, and 10.000 ng/ml. The specificity of the protein microarrays was tested against BSA, TNFα, and IL-8 which were diluted in reagent diluents. Fluorescence intensities were quantified by the software Signalyse^{TM}(Holger Klapproth Life Science, Germany).

2.4. Instrumentation

Protein microarrays were produced with a contactless dispenser (SciFlexArrayer S3, Scienion AG, Germany) and after completion of the print irradiated with UV-light (Stratagene Europe, Amsterdam, The Netherlands). Read-out of microarrays was performed using a SensoSpot©-Sensovation's fluorescence array imaging reader (Germany).

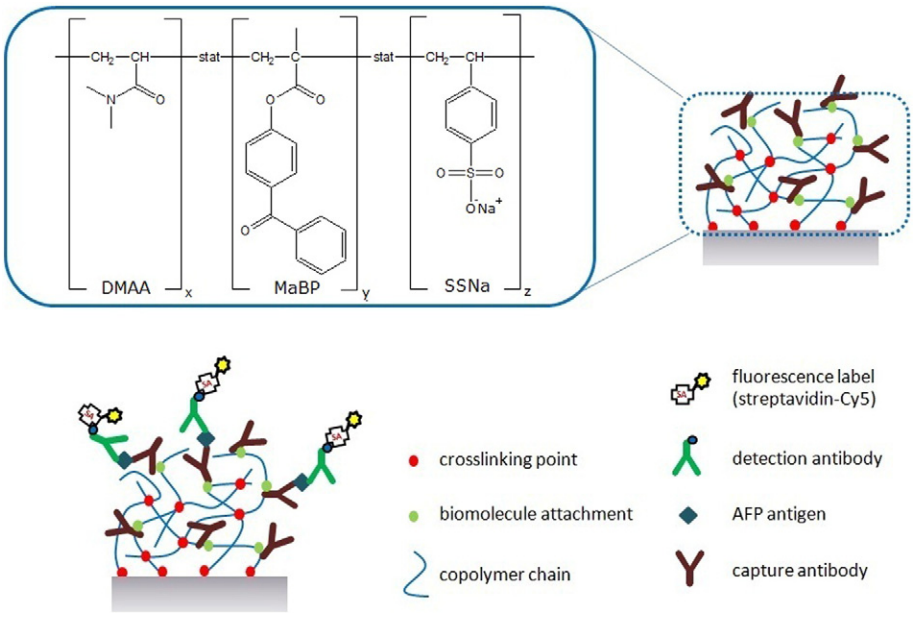

Fig. 1. Schematic depiction of the chemical structure of the copolymer used for the microarray fabrication (upper) and the platform of AFP detection (lower). The photoactive benzophenone groups are used to simultaneously crosslink the polymer, bind the biomolecule, and attach the forming hydrogel to the surface.

Fig. 2. Schematic depiction of the biochip layout. The 1st column was printed with streptavidin-Cy5 at a ratio of 1:200 as a coupling control (CC). The 2nd, 3rd, 4th, and 5th columns were spotted with 10, 50, 100, and 200 μg/ml of capture antibody (Cap.), respectively. The 6th was generated as detection control (DC) which is detection antibody. The 7th column was printed as negative control (NC) which is a printing solution without protein. Each column consists of 6 identical spots.

3. Results and discussion

3.1. Protein microarray fabrication

The protein solutions in PBS buffer were mixed with the photoactive copolymer and printed onto PMMA substrate using a piezo printer. The copolymer consists of poly(dimethylacrylamide) (PDMAA) as a hydrophilic matrix component. Sodium styrenesulfonate (SSNa) repeat units are incorporated to introduce charges to the polymer and enhance solubility of the final copolymer in water and MaBP to provide photo reactive benzophenone groups to the polymer [17]. After printing of the polymer/protein mixtures, the dispensed drops dry rather rapidly. During irradiation with UV-light the benzophenone units become activated into a biradicaloid triplet state and the reactive intermediate undergo C,H insertion reactions. In the course of this process three reactions occur simultaneously [18]: 1) transformation of the water soluble polymer into a hydrogel, 2) covalent linking of the forming hydrogel to the substrate and 3) immobilization of the protein added to the printing solution to the hydrogel via covalent binding [15,17].

The amount of capture antibody bound to the sensor surface can be controlled by varying the concentration of proteins in the printing solution and the volume of the deposited drops. Moschallski et al. [15] have shown that a higher binding capacity was obtained in the hydrogels when compared with the monolayer [15].

3.2. AFP detection

A schematic depiction of the AFP detection is shown in Fig. 1. After immobilization of the capture antibody as described above and incubation with the analyte solution, the latter was detected by conjugation with a biotinylated anti-AFP antibody and streptavidin-Cy5 conjugation. Fluorescence images of chips after incubation with solutions of various AFP concentrations are shown in Fig. 3. The coupling control (CC; 1st column) and detection control (DC: 6th column) were used to check the immobilization process. The negative control does not yield any fluorescence signal (NC; 7th column) and is used to obtain the background signal.

The results in Fig. 3 showed that at a concentration of 0.010 ng/ml of AFP a clear signal could be observed for spots printed at a concentration of 200 μg/ml capture antibody (5th column). When the capture antibody concentration was lower (100 and 50 μg/ml) (4th and 3rd column) the lowest concentration at which signals could be observed were 0.025 and 0.050 ng/ml of AFP, respectively. For even lower concentration of the spotting solution, (10 μg/ml capture antibody (2nd column)), the obtained fluorescence spot images were still visible, but could no longer be used for protein quantification.

3.3. Calibration curve

The highest signal intensity of the fluorescence images could be obtained on spots generated from 200 μg/ml capture antibody solutions and decreased for lower antibody concentrations (100, 50 and 10 μg/ml). This indicated that antibodies are immobilized in the flexible, highly swollen hydrogel, which allows easy access of the antigens to the immobilized antibody. The fluorescence signals obtained from 10 μg/ml capture antibody were only rather weak and accordingly had a high noise level. For all other spot locations the fluorescence signal seemed to be saturated at about 1 to 2 ng/ml of AFP. The highest detection slope of the fluorescence signal as a function of the AFP concentration and thus highest sensitivity was obtained on spots generated from 200 μg/ml capture antibody (Fig. 4). This indicates that the surface concentration of the capture proteins in the hydrogel spots is still not so high that the binding capacity is becoming compromised.

3.4. Limit of detection

To determine the limit of detection of the protein microarray biochip, signal-to-background ratio (SBR) was determined as a function of the antibody concentration during chip printing. When a comparison of the results of the chip analysis with the concentration range relevant for diagnostic is made, it should be noted that for all following experiments the solutions were diluted by a factor of 1:100. The SBR in these experimental series was defined as the quotient of fluorescence intensities of probe containing hydrogel and empty spots. Fig. 5 shows the SBR of 3 different biochips measured at concentrations of 0.005, 0.010, 0.025, and 0.050 ng/ml of AFP antigen. The results indicated that the limit of detection of the biochip (at least for the higher antibody concentrations) was 0.010 ng/ml and the limit of quantification 0.050 ng/ml for which an SBR above 10 is required. For maximal response a concentration of the capture antibody of 200 μg/ml should be chosen.

3.5. Specificity of the protein microarray biochip

To evaluate the specificity of this biochip, AFP determination was carried out in the presence of 2 cytokines (TNFα and IL-8) and a typical blood protein (BSA). These proteins were added as model compounds (0.1 ng/ml) to check for possible interference with other proteins present in the analyte. As shown in Fig. 6 the addition of TNFα, IL-8 and BSA resulted only in a very small increase of the background fluorescence signal. This is not surprising as it has been shown, that such surface-

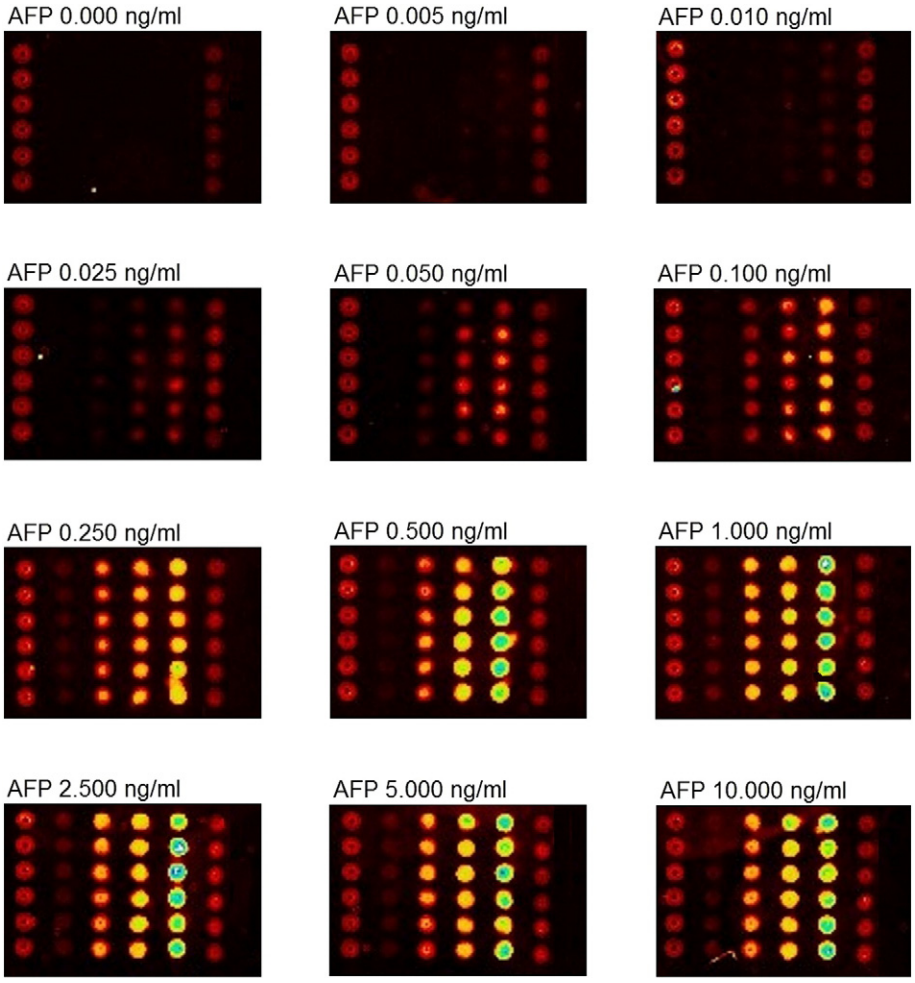

Fig. 3. Fluorescence images biochips after exposure to various AFP concentrations: 0.0, 0.005, 0.010, 0.025, 0.050, 0.100, 0.250, 0.500, 1.000, 2.500, 5.000, and 10.000 ng/ml.

attached neutral hydrogels are strongly protein repellent and prevent the unspecific adsorption of proteins [19]. Accordingly, the ratio between specific and non-specific signal still remained almost at a factor of 55 for the mixed molecules so that any potential interference of the added proteins during AFP quantification can be neglected.

4. Conclusion

In this study, a rapid photo-immobilization process based on C,H insertion reactions was successfully applied to fabricate protein microarrays for the detection and quantification of alpha fetoprotein (AFP).

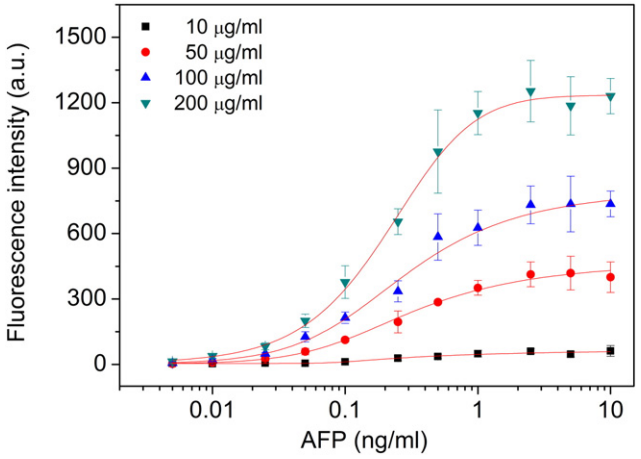

Fig. 4. Relationships between fluorescence intensity and AFP concentrations on different surface density of capture antibody: 10 μg/ml (black square), 50 μg/ml (red circle), 100 μg/ml (blue up-pointing triangle) and 200 μg/ml (green down-pointing triangle).

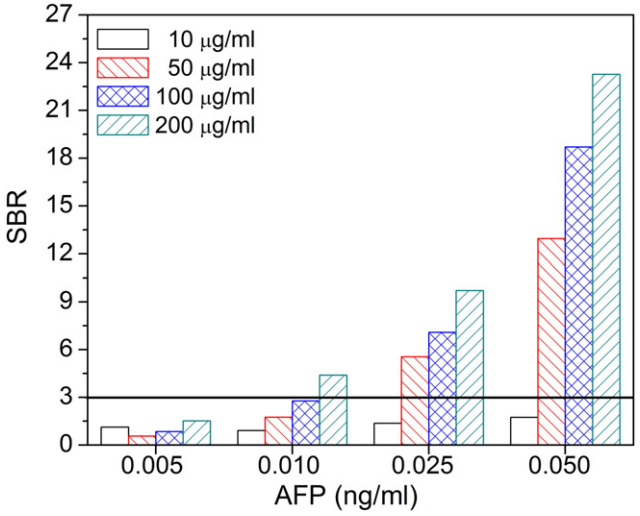

Fig. 5. Signal-to-background ratios (SBR) indicating the limit of detection value (above SBR = 3) of chips printed with solution containing 10, 50, 100, and 200 μg/ml capture antibody.

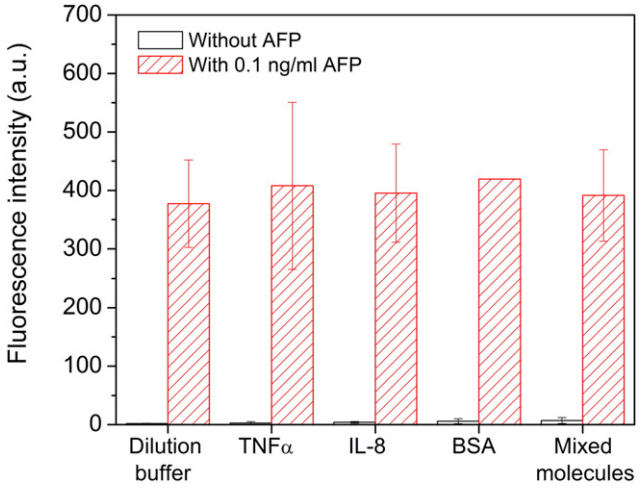

Fig. 6. Specificity of protein microarray biochip toward AFP antigen (0.1 ng/ml) against other biomolecules (0.1 ng/ml): TNFα (tumor necrosis factor alpha), IL-8 (interleukin) and BSA (bovine serum albumin).

Microarray printing of a mixture of probe molecules and photopolymer followed by brief UV-exposure is sufficient to generate protein microarrays, which allow quantification of AFP over the whole concentration range required in diagnostics, i.e. between 5 and 100 ng/ml. The sensitivity of the developed chip is so high that all analyte solutions need to be diluted by 100 times in order to avoid overloading of the surface, which would prevent quantification. After dilution a linear response between concentration and read-out signal was observed.

An additional interesting aspect of the developed method is that unmodified plastic chips can be used as substrates so that the array generation is fast and inexpensive. Currently studies are under way which aim at the integration of further markers on the chip and on the validation of the produced chips in diagnostic tests performed in a clinical setting.

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