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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).

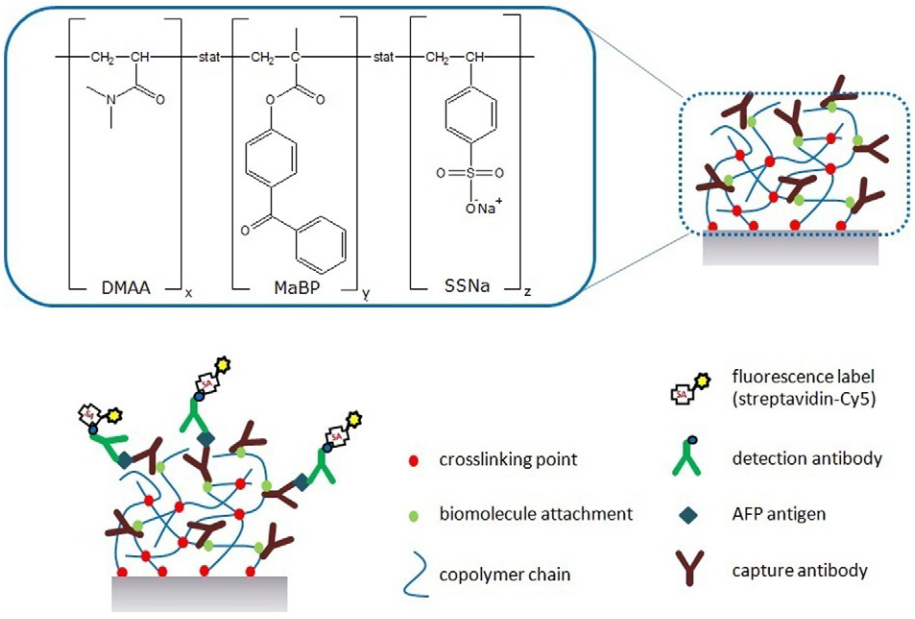

Fig. 1. Schematic depiction of the chemical structure of the copolymer used for the microarray fabrication (upper) and the platform of AFP detection (lower). The photoactive benzophenone groups are used to simultaneously crosslink the polymer, bind the biomolecule, and attach the forming hydrogel to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