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New platforms for 3-D microarrays: Synthesis of hydrophilic polymethacrylate monoliths using macromolecular porogens

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ABSTRACT

Hydrophilic macroporous monolithic material based on poly(glycidyl methacrylate-co-glycerol dimethacrylate) was synthesized by photo-initiated free-radical polymerization. Different pore-forming agents, including low molecular mass cyclohexanol and dodecanol, as well as the solutions of hydrophobic polymers, namely, polystyrene in toluene and poly(dimethyl siloxane) in hexane, were used to obtain the macroporous polymer platforms intended for new type of 3-D microarrays (biochips). The porous characteristics of functional copolymers obtained were investigated by mercury intrusion porosimetry and scanning electron microscopy. Some of developed materials were tested for protein microarray construction. Demonstration of potential of suggested materials, as well as optimization of protein covalent immobilization conditions, were realized using model mouse IgG – goat anti-mouse IgG affinity pair.

1. Introduction

Comparatively recently introduced to biomedical practice *microarrays* (biochips) have expanded rapidly to the major fields of mentioned discipline [1]. In fact, these miniaturized devices represent the modern generation of well-plate test-systems with much more possibilities and much higher analytical sensitivity. According to the latter, these systems can be counted as micro or nanoanalytical scale tools. The application of these modern devices gives a possibility of parallel analysis and screening of multicomponent biological samples. Thus, biochips allow simultaneous analysis of several hundreds or even thousands compounds.

The application of discussed test-systems for supersensitive analysis of any biosubstances (including proteins) is based on a specific binding of target biomolecule to its biocomplementary ligand immobilized on a solid surface. It is known that the efficiency of processes based on a biorecognition principle strongly depends on the properties of used solid support. An "ideal" matrix intended for protein microarray has to possess the following criteria: (a) to be insoluble and protein friendly; (b) to be easily manufactured; (c) to have high chemical stability at analytical conditions; an appropriate matrix also has to provide: (d) stable attachment of protein ligand to a solid surface; (e) low level of side-proteins adsorption; (f) good spot (loading ligand zone) uniformity; (g) high specific adsorption capacity, that means high sensitivity of analysis, and, finally, (h) high compatibility with microarray related devices [2].

Two immobilization strategies are available for protein ligands, namely, physical adsorption or covalent binding [3]. Both processes provide rather uncontrolled attachment. However, if the covalent binding is stable and mostly irreversible, the adsorption may be followed by ligand leakage during washing steps, or as a result of exchange of adsorbed protein molecules with the surrounding protein sample.

The covalent attachment seems to be more preferable for ligand's immobilization on the surface, but the chemistry used for affinity matrix preparation meets some requirements. For instance, to prevent any loss of activity of attached biomolecule, the reaction of covalent immobilization must proceed at mild conditions and produce friendly side-products [2]. In fact, most of materials used for microarray construction do not possess the highly reactive groups and have to be preliminary activated that, in turn, complicates the procedure of chip manufacturing.

Biological microarrays can be realized in two formats. First of them is based on the use of so called two-dimensional (2-D) supports. In this case, the protein of interest reacts with a ligand located at a rigid monolayer representing microarray's surface. Today such devices based on functionalized glass slides, non-porous synthetic polymers or metals dominate in practical applications [4,5]. Three-dimensional matrices (3-D microarrays) include polyacrylamide gels [5–7], agarose [8], dextran gel [9] and nitrocellulose films [10,11]. The 3-D devices formed on a rigid inert surface, usually glass, are porous. Thus, the ligands (proteins)

Abbreviations: GMA, glycidyl methacrylate; EDMA, ethylene dimethacrylate; GDMA, glycerol dimethacrylate; CyOH, cyclohexanol; DoOH, dodecanol; PS, polystyrene; PDMS, poly(dimethyl siloxane); 2-D and 3-D, two- and three-dimensional, respectively; BSA, bovine serum albumin; IgG, immunoglobulin G.

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penetrate by molecular diffusion into 3-D space and react with functional groups of intraporous surface. The 3-D microarrays have much larger surface area comparatively to 2-D matrices allowing immobilization of much bigger amount of protein and, consequently, detection of much higher signal intensities after affinity adsorption of marked target protein. Moreover, 3-D materials are characterized by reduced protein denaturation because of homogeneous aqueous environment and low non-specific adsorption. However, in comparison with 2-D supports, the most of existing 3-D materials are difficult to be fabricated. Furthermore, the washing step of 3-D, for example, gel-based, matrices is more time-consuming because of diffusion-controlled mass-exchange of big biomolecules with low molecular diffusivity.

In the early 1990s a new class of chromatographic sorbents, namely, *continuous media* based on rigid macroporous polymer monoliths, was introduced [12,13]. These materials are synthesized by free-radical bulk copolymerization of functional monomer and cross-linker in a presence of porogenic solvents and characterized by fixed porous properties even in a dry state [14,15]. Their internal structure consists of interconnected system of polymer microglobules separated by pores, and their structural rigidity is secured by extensive cross-linking.

One of significant advantages of such materials is their simple synthesis. Porous polymer monoliths can be formed in situ into any shape [16]. Moreover, the possibility of introduction of reactive groups at polymerization step allowing further one-step protein immobilization is another positive feature. Today, these materials are successfully applied in many fields, such as liquid and gas chromatography (HPLC) [17,18], electrochromatography [19], efficient flow-through enzyme reactors [20], microfluidics [21], etc. Despite wide practical application, polymer monoliths still represent the object of extensive and thorough scientific investigations.

Recently, the idea of development of a new type of 3-D microarrays based on rigid macroporous monolithic materials has been realized by our group. The well known from affinity chromatography on monoliths macroporous copolymer of glycidyl methacrylate (GMA) and ethylene dimethacrylate (EDMA) [poly(GMA-*co*-EDMA)] bearing in its structure the original epoxy groups was studied for nanoanalytical detection of influenza virus in biochip format [22]. In this work for the first time a quantitative comparison of affinity pair formation at flow-through (affinity chromatography) and non-flowing (microarray) conditions was carried out. Additionally, the different ways of protein immobilization onto the poly(GMA-co-EDMA) macroporous layers using intermediate surface modification were also studied [23].

This paper is devoted to the synthesis and investigation of new monolithic material based on GMA and hydrophilic glycerol dimethacrylate (GDMA) [poly(GMA-co-GDMA)] intended for protein analysis using microarray format. It is well known that EDMA represents the most popular cross-linking agent used for the preparation of rigid macroporous methacrylate monolithic polymers and most often copolymerized with butyl methacrylate, glycidyl methacrylate, hydroxyethyl methacrylate, etc. [14]. As to GDMA, there is a few publications corresponding to the synthesis of macroporous monolithic columns for HPLC based on poly(butyl methacrylate-co-glycerol dimethacrylate) [24] and poly(glycerol dimethacrylate) [25] materials. Being hydrophilic, GDMA allows the construction of protein friendly polymer surface provided the better penetration of biomolecules into 3-D porous structure. Moreover, the polymer hydrophilization allows the better surface wetting that also favors to a bioanalytical performance. In this study, the search of appropriate porogenic solvents was carried out to optimize the porous structure of resulting poly(GMA-co-GDMA) matrix. The optimal parameters of synthesis, such as time of polymerization and concentration of initiator, were also established. The efficiency of developed materials for protein microarray was tested using model affinity pair, namely, mouse immunoglobulin G (IgG) – anti-mouse goat IgG. The results obtained were compared with those revealed for earlier developed and widely used poly(GMA-co-EDMA) material.

2. Experimental part

2.1. Materials and instruments

Glycidyl methacrylate (GMA, 97% pure), glycerol dimethacrylate (GDMA, 85% pure), ethylene dimethacrylate (EDMA, 98% pure), 2-hydroxy-2-methylpropiophenon (Darocur-1173, 97% pure), dodecanol (99% pure), polystyrene (MM_w 382 000) and poly(dimethyl siloxane) (MM_w 90 000) were purchased from Sigma–Aldrich Rus (Moscow, Russia). Toluene, hexane, ethanol and other organic solvents were obtained from Vecton Ltd. (St. Petersburg, Russia). Purified mouse IgG was the product of Zytomed GmbH (Berlin, Germany), goat anti-mouse IgG conjugated with Alexa Fluor 555 was purchased from Molecular Probes (Eugene, USA). TopBlock was a product of Fluka AG (Buchs, Switzerland). Following buffers were used for microanalytical manipulations: 0.01 M sodium borate buffer (pH 9.4), 0.01 M sodium phosphate buffer, containing 0.15 mol/l NaCl (PBS, pH 7.4) and 0.3 M sodium citrate buffer, containing 3 mol/l NaCl and 2% sodium dodecyl sulfate (SSC, pH 7.0). All buffers were prepared by dissolving the analytical grade salts in distilled water and were additionally purified by filtration through a 0.45 μm Milex Millipore microfilter (Wien, Austria). The glass slides of $25 \times 75 \times 1.2$ mm dimensions were obtained from BioVitrum (Saint-Petersburg, Russia).

The 125 W mercury lamp (Philips, Netherlands) of wide radiation spectrum and constant intensity was used for free-radical polymerization. The monolith morphology was studied using scanning electron microscope JSM-35 CF JEOL (Tokyo, Japan). The mean pore size and pore size distribution were determined by mercury intrusion porosimetry using PASCAL 440 Thermoquest Instrument (Italy).

For spotting of proteins to be immobilized on a microarray surface sciFLEXARRAYER S3, Scienion (Berlin, Germany) was used. The procedure of biochip washing was carried out using a Thermomixer Comfort (Eppendorf, Germany). The special secure seal hybridization chambers (Grace Biolabs, Bend, USA) were used to perform the coupling of immobilized mouse IgG with goat anti-mouse IgG conjugated with Alexa Fluor 555. The microarrays were scanned using Scanner GenePix 4000 B (Axon Instruments, USA). GenePix 6.0 software was used to analyze the data.

2.2. Methods

2.2.1. Synthesis and characterization of poly(GMA-co-GDMA) monoliths

The mixture containing monomers, porogens and 2-hydroxy-2-methylpropiophenon (Darocur-1173) as initiator was applied for synthesis of polymer monolithic layers. The proportions functional monomer: cross-linker and porogens: monomers were equal to 60:40 vol% in both cases. A copolymerization process was optimized by variation of a concentration of initiator in a range of 0.2–1.2 mass%. To find appropriate composition of porogenic solvents, the mixtures with different combinations and ratios of cyclohexanol, dodecanol and solutions of polystyrene (PS) in toluene and poly(dimethyl siloxane) (PDMS) in hexane were applied. All details concerning variation of porogens composition are presented in Table 1. All reagents for polymerization were mixed and the polymerization mixture was purged with nitrogen for 5 min. Then the mixture was placed into the well of $20 \times 60 \times 0.2$ mm preliminary prepared via mechanical treatment