Synthesis and Investigation of a New Macroporous Monolithic Material Based on an *N*-Hydroxyphthalimide Ester of Acrylic Acid-*co*-Glycidyl Methacrylate-*co*-Ethylene Dimethacrylate Terpolymer

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Received 11 March 2008; accepted 24 June 2008
DOI 10.1002/app.29130
Published online 13 October 2008 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: A macroporous monolithic material based on an *N*-hydroxyphthalimide ester of acrylic acid-*co*-glycidyl methacrylate-*co*-ethylene dimethacrylate terpolymer was synthesized by photoinitiated free-radical polymerization. Several porogenic solvents, such as cyclohexanol, dodecanol, and poly(ethylene glycol)s, were tested to obtain the monolithic material with an optimal pore size allowing unrestricted penetration of large molecule (proteins) into a three-dimensional porous space. The new monolithic material was covalently bound to an inert surface (glass) directly in the polymerization step, and it was suggested as a solid matrix for the development of new types of three-dimensional protein microarrays (biochips). A demonstration of the potential of the suggested microarray platform as well as optimization of microarray performance conditions was realized with a model mouse immunoglobulin G/goat anti-mouse immunoglobulin G affinity pair. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 111: 692–700, 2009

Key words: nanotechnology; proteins; radical polymerization; supports

INTRODUCTION

Protein microarrays (biochips) based on a principle of biorecognition represent independent and quite promising analytical tools with great potential in different practical fields, such as molecular biology, medicine, analytical biotechnology, pharmacology, ecology, and bioinformatics.[1] It is known that the efficiency of processes based on bioaffinity interactions depends directly on the properties of the solid support. An ideal matrix intended for affinity interactions has to satisfy certain requirements, such as insolubility, absence of side protein adsorption, and high chemical stability under the analytical conditions.[2] Furthermore, the immobilization of an affinity ligand on a chosen solid surface represents an important step of biochip construction. In the case of protein analysis, care must be concentrated on the minimization of any *in vitro* manipulations with proteins to save their activity and specificity. Therefore, analytical methods have to be fast to prevent protein denaturation or even degradation.

A new class of so-called continuous media based on rigid macroporous polymer monoliths was introduced in the early 1990s.[3,4] These materials are characterized by fixed porous properties, even in a dry state. Their internal structure consists of interconnected systems of polymer microglobules separated by pores, and their structural rigidity is secured by extensive crosslinking. Numerous excellent reviews on the preparation of monolithic media were published in the last decade. Among them, we can adduce only two, but they are the most recent ones.[5,6] Today, these materials are successfully applied in high-performance liquid chromatography[7–10] and bioconversion processes (flow-through enzyme reactors),[11,12] capillary electrochromatography,[13] gas chromatography,[14] microfluidics,[15] catalysis,[6,16] solid-phase extraction,[17] and supported organic reactions.[18,19] Despite such widespread practical applications, polymer monoliths still remain the object of extensive and thorough scientific investigation.

Most frequently, macroporous monolithic polymers used for the preparation of bioaffinity sorbents are copolymers of reactive glycidyl methacrylate (GMA), particularly the well-studied copolymer poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) [poly(GMA-*co*-EDMA)].[20,21] The original epoxy groups of this support allow a wide range of chemical reactions including those used for sorbent

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Contract grant sponsor: Russian Science Support Foundation (to E.G.V.).

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biofunctionalization, such as covalent binding of biological molecules complementary to the dissolved partner to be isolated.[2] These efficient stationary phases have numerous important advantages, such as mechanical and chemical stability, insignificant nonspecific protein adsorption, and the presence of original epoxy groups in the copolymer chemical structure allowing a one-step reaction with an amino-bearing ligand (protein).

Recently, a new type of three-dimensional protein microarray intended for the nanoanalytical detection of the influenza virus and based on rigid macroporous poly(GMA-*co*-EDMA) monolithic operative layers was developed by our scientific group.[22] In this study, for the first time, a quantitative comparison of affinity pair formation under flow-through (affinity chromatography) and nonflowing (microarray) conditions was carried out. Despite the numerous positive features of this popular material, it has one disadvantage, namely, very slow kinetics of ligand covalent attachment via sorbent epoxy groups (16–18 h).[16,23] Obviously, the total rate of the immobilization process is limited both by the rate of protein (ligand) diffusion into the pores and by the rate of the chemical reaction between the polymer functional groups and amino-bearing ligand. To increase the rate of the immobilization process and, consequently, the affinity adsorption capacity of the monolithic material, different methods of additional surface modification can be used. For example, the introduction of aldehyde, imidazole carbamate, and succinimide carbonate groups by the chemical conversion of hydroxyls generated via the controlled hydrolysis of original epoxy groups can provide much faster immobilization and a higher affinity binding capacity of the obtained material.[24] However, the realization of multistep solid-phase chemical modifications represents a complicated and nontechnological method of enhancing surface reactivity.

The most convenient way of getting the desirable surface chemistry of monolithic materials is a direct copolymerization of the functional monomer(s) with a crosslinking agent. However, some acrylate and methacrylate monomers with attractive functionalities are solid substances. Thus, to prepare a homogeneous polymerization mixture, it is necessary to dissolve such monomers in an appropriate solvent. The solvent used for this purpose has to be thermodynamically good both for the monomer to be used and for the resulting polymer. The presence of a significant amount of a good solvent in the polymerization mixture will not allow a monolithic material with sufficient porous characteristics to be obtained. This problem can be solved by the introduction of a third comonomer with desirable properties into the structure of an appropriate monolithic material. Such an approach is widely described in the current literature. Hahn et al.[25] reported an original approach to the preparation of affinity monolithic sorbents via the direct triple copolymerization of glycidyl methacrylate (GMA), GMA conjugated with a preliminary synthesized peptide, and ethylene dimethacrylate (EDMA).[25] A similar method for introducing a novel functionality, namely, [2-(methacryloyloxy)ethyl]trimethyl ammonium, into the known poly(GMA-*co*-EDMA) material was reported by Bedair and El Rassi.[26] In all these cases, GMA was not considered a reactive monomer but was used to maintain a sufficient morphology of the macroporous poly(GMA-*co*-EDMA) monoliths.

This study was devoted to the synthesis and investigation of a new monolithic material based on a terpolymer of *N*-hydroxyphthalimide ester of acrylic acid (HPIEAA), GMA, and EDMA [poly (HPIEAA-*co*-GMA-*co*-EDMA)] for protein analysis with a microarray format. The presence of highly reactive activated ester groups allowed the easy covalent attachment of amino-bearing ligands (proteins). A search for appropriate porogenic solvents and their compositions in a reaction mixture was carried out to optimize the porous structure of the resulting terpolymer.

EXPERIMENTAL

Materials and instruments

GMA (97% pure), EDMA (98% pure), cyclohexanol (CyOH; 99% pure), dodecanol (DoOH), poly(ethylene glycol)s with molecular masses of 400 and 600 (PEG-400 and PEG-600, respectively), 1,4-dioxane, and bovine serum albumin (BSA) were purchased from Sigma–Aldrich GmbH (Taufkirchen, Germany). 2-Hydroxy-2-methylpropiophenone (Darocur-1173; 97% pure), used as an initiator, was obtained from Merck-Schuchard (Hohenbrunn, Germany). HPIEAA was synthesized from acryloyl chloride and *N*-hydroxyphthalimide in the presence of triethylamine in tetrahydrofuran according to a method published elsewhere.[27] Purified mouse immunoglobulin G (IgG) was obtained from Zytomed (Berlin, Germany); goat anti-mouse IgG conjugated with Alexa Fluor 555 was purchased from Molecular Probes (Eugene, OR). TopBlock was purchased from Fluka AG (Buchs, Switzerland). The following buffers were used for microanalytical manipulations: sodium borate buffer ($1.25 \times 10^{-2} M$ $Na_2B_4O_7 \cdot 10H_2O$ and $8.8 \times 10^{-3} M$ NaOH), phosphate-buffered saline (PBS; $0.137M$ NaCl, $2.7 \times 10^{-3} M$ KCl, $4.3 \times 10^{-3} M$ Na_2HPO_4, and $1.4 \times 10^{-3} M$ KH_2PO_4), and sodium chloride-sodium citrate buffer (SSC; $3M$ NaCl and $0.3M$ $Na_3C_6H_5O_7$) containing 2% sodium dodecyl sulfate. All buffers were prepared by the dissolution of analytical-grade salts in distilled water and were additionally

purified by filtration through a Milex 0.45-μm microfilter (Millipore, Wien, Austria).

A 125-W mercury lamp (Philips, Eindhoven, The Netherlands) with a wide radiation spectrum and constant intensity (1.5 mW/cm^2) was used for the free-radical copolymerization of the chosen monomers. A Fourier transform infrared spectrophotometer (Brucker, Ettingen, Germany) was used for the verification of the presence of the HPIEAA comonomer in the final terpolymer.

The monolithic porous structure was studied with a JSM-35 CF scanning electron microscope (Japan Electron Optic Co. Ltd, Tokyo, Japan). Gold was preliminarily sputtered onto the polymer samples (the coating thickness was ca. 100 Å) with a Polaron scanning electron microscopy (SEM) coating system (Polaron Equipment, Ltd., Watford, England). The porous properties of the prepared materials were determined by mercury intrusion porosimetry with a Pascal 440 (ThermoQuest Instrument, Rodano, Italy). The polymer samples with a mass of ~ 100 mg were analyzed in the dry state.

For the spotting of immobilized proteins, Arraer 417 (Affimetrix, Santa Clara, CA) was used. The biochip washing was carried out with a Thermomixer comfort (Eppendorf, Hamburg, Germany). Special secure-seal hybridization chambers (Grace Biolabs, Bend, OR) were used to carry out the coupling of immobilized mouse IgG to goat anti-mouse IgG conjugated with Alexa Fluor 555. Resulting fluorescent zones on a microarray were scanned with a GenePix 4000 B scanner (Axon Instruments, Foster City, CA). GenePix 6.0 software was used to quantify the scan data.

Methods

Preparation and characterization of the poly(HPIEAA-*co*-GMA-*co*-EDMA) monoliths

To prepare the samples of the terpolymer under discussion, polymerization mixtures containing the comonomers (HPIEAA, GMA, and EDMA), different porogenic solvents in defined proportions, and the initiator Darocur-1173 were used. The proportions of the comonomers were kept constant in all experiments and were equal to 13.3 mol % HPIEAA, 53.3 mol % GMA, and 33.3 mol % EDMA. The copolymerization process was optimized by the variation of the concentration of the initiator in the range of 0.2–2.0 mass %. The monomers and chosen porogenic solvent were mixed in two volume ratios, namely, 40 : 60 and 25 : 75. To determine the appropriate composition of the porogenic solvents, mixtures with different combinations and ratios of CyOH or DoOH to PEG-400 and PEG-600 were tested. All details concerning the variation of the porogen composition are presented in Table I. After bubbling with nitrogen for 5 min (to remove the dissolved oxygen), the polymerization mixture was introduced into a special round-shaped plastic container 4 mm high and 20 mm in diameter. The reaction time was varied from 3 to 20 min. To investigate the kinetics of the polymerization process, the following experimental conditions were used: an initiator concentration of 1%, a porogen/monomer ratio of 75 : 25, and a DoOH/PEG-600/dioxane ratio of 29 : 29 : 42. The dependence of the monomer conversion on the initiator concentration

TABLE I
Composition of the Polymerization Mixture Used for the Preparation of the Poly(HPIEAA-*co*-GMA-*co*-EDMA) Monoliths

Monolith	Porogen mixture (%, w/w)					Porogen/monomer ratio (%, w/w)
	1,4-Dioxane	CyOH	DoOH	PEG-400	PEG-600	
M1	42	58.0				60 : 40
M2	42				58.0	60 : 40
M3	42	5.8			52.2	60 : 40
M4	42	40.6	17.4			60 : 40
M5	42	29.0	29.0			60 : 40
M6	42	17.4			40.6	60 : 40
M7	42	29.0			29.0	60 : 40
M8	42		34.8		23.2	60 : 40
M9	42		29.0		29.0	60 : 40
M10	42		23.2		34.8	60 : 40
M11	42		23.2	34.8		60 : 40
M12	42		17.4		40.6	60 : 40
M13	42		29.0		29.0	75 : 25
M14	42		23.2	34.8		75 : 25
M15	42		34.8	23.2		75 : 25
M16	42		17.4	40.6		75 : 25
M17	42		29.0	29.0		75 : 25
M18	42		23.2		34.8	75 : 25

was examined with a polymerization mixture with a porogen/monomer ratio of 75 : 25 and a DoOH/PEG-600/dioxane ratio of 29 : 29 : 42. An irradiation time of 30 min was used in these experiments. After the polymerization process was finished, the obtained monolithic materials were washed with ethanol and chloroform to remove the porogens and other unreacted soluble compounds and dried at 50°C until the sample mass was constant. After that, the polymer samples were weighed, and the polymerization yield was calculated.

The presence of HPIEAA in the final polymer was determined with IR spectroscopy. For this purpose, the samples consisted of 5 mg of finely dispersed polymer, and 600 mg of KBr was applied. The spectra were detected from 200 to 4000 cm^{-1}.

Microarray platform fabrication

The first step of biochip construction was selective etching of a glass surface with hydrofluoric acid. The process was carried out with intensive interfusion and resulted in the formation of special wells of a desirable shape (rectangular, 18 mm × 50 mm) on the glass slide surface. A special paraffin mask was used in this step. Then, the manufactured slides were washed three times with water, boiled in 0.1*M* NaOH for 40 min, washed again three times with distilled water, and finally dried at 100°C.

To form double bonds necessary for further copolymerization, glass slides were held in a 15% solution of 3-(trimethoxysilyl) propyl methacrylate in dry toluene for 17–20 h at room temperature. To avoid the photochemical destruction of the used silane, a reactor was preliminarily wrapped in aluminum foil. The functionalized glasses were washed three times with toluene, acetone, and ethanol and stored in ethanol in the dark. The glasses were dried for 1.5 h at 35°C directly before use in polymerization.

The polymerization mixture was applied to preliminarily prepared and functionalized wells on the glass surface, and the polymerization was allowed to proceed at room temperature in a nitrogen medium.

Performance of microanalysis with a model affinity pair of mouse IgG and goat anti-mouse IgG conjugated with Alexa Fluor 555

Immobilization of mouse IgG on the monolith surface. To establish the optimal conditions of the immobilization process, the pH and concentration of the mouse IgG solution were varied.

Mouse IgG was always spotted in 10 replicates in one column on the surface of poly(HPIEAA-*co*-GMA-*co*-EDMA). Two printing buffers were used for the immobilization of mouse IgG, namely, a sodium borate buffer (pH 9.5) and PBS (pH 7.5). The time of ligand immobilization corresponded to 2 h.

Washing procedures and surface blocking

After the immobilization process was stopped, the slides were washed with PBS, NaCl, and once again PBS for 40 min for each solution. Then, the slide surface was blocked with a 1% BSA solution in PBS for 45 min with slight stirring. The ready-to-analyze biochips were washed with PBS for 5 min and then two times with water for 5 min.

Coupling with anti-mouse IgG and quantification. Probes of goat anti-mouse IgG conjugated with Alexa Fluor 555 with a concentration of 200 ng/mL were prepared by the dilution of a commercial solution with a concentration of 2 mg/mL in TopBlock. Slides with immobilized mouse IgG were incubated with the obtained solution at 25°C and 300 rpm for 2 h. After coupling, the slides were washed with the following washing buffers: 2×SSC (3*M* NaCl and 0.3*M* sodium citrate) containing 2% sodium dodecyl sulfate (pH 7.0) for 5 min, 1×SSC (pH 7.0) for 5 min, and 0.5×SSC (pH 7.0) for 5 min.

Then, the slides were dried, and fluorescent zones were scanned with the same gain for all arrays. The signal intensity (signal mean), relative signal intensity (signal mean − background mean), and signal/noise ratio [(signal mean − background mean)/standard deviation of background] were calculated.

RESULTS AND DISCUSSION

As noted previously, the main goal of this study was the development of a new type of highly reactive macroporous monolithic material based on a ternary copolymer containing functional groups allowing the fast covalent immobilization of amino-bearing ligands (proteins). An important step of this study was the optimization of the polymerization conditions to prepare thin macroporous monolithic layers with a pore size that could provide both unrestricted penetration of large protein molecules into the three-dimensional porous space and, at the same time, sufficient adsorption capacity of the resulting porous surface.

HPIEAA (Fig. 1) was chosen as the functional monomer. The activated ester group appears to be very active in the reactions with proteins. However, HPIEAA is a crystalline substance and has to be dissolved for its introduction into a polymerization mixture. The solvent used for this purpose becomes a component of the porogen mixture and surely influences the formation of the macroporous structure. Obviously, if the chosen 1,4-dioxane satisfactorily dissolves HPIEAA, the polymer molecules containing this monomer will be also solvated by this solvent. If HPIEAA were applied as the only functional monomer, it would be impossible to obtain a macroporous monolithic material with the

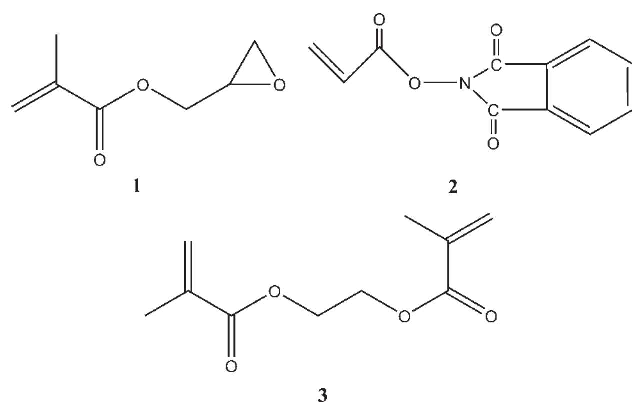

Figure 1 Chemical structures of the comonomers: (1) GMA, (2) HPIEAA, and (3) EDMA.

desired properties. To obtain an HPIEAA-containing monolith with morphologically peculiar properties close to those of the best material for applications based on affinity interaction [i.e., poly(GMA-*co*-EDMA)], the substitution of the GMA part with HPIEAA was chosen as the most appropriate. In this case, GMA is not considered a functional comonomer because the interaction between the *N*-hydroxyphthalimide (HPIE) groups of the material and protein amino groups proceeds extremely fast versus the reaction between amino and epoxy groups.

The volume of 1,4-dioxane necessary for dissolving HPIEAA in the amount required for polymerization was equal to one-fourth of the total volume of the polymerization mixture. It was assumed that such a significant amount of an undesirable solvent could lead to serious difficulties in the formation of a material with desirable porous properties. Therefore, in this study, care was taken to investigate the influence of the nature and composition of the porogenic solvents on the average pore size and pore size distribution of the final polymer product.

The obtained poly(HPIEAA-*co*-GMA-*co*-EDMA) materials were investigated with IR spectroscopy. The appearance of two adsorption bands at 1788 and 1817 cm^{-1} corresponding to valence oscillations of ester and imide carbonyls reliably proved the introduction of HPIEAA into the copolymer structure.

Dependence of the porous properties of the monoliths on the properties of the porogenic solvents

To obtain a polymer monolithic material with an average pore size providing unrestricted penetration of large protein molecules into the three-dimensional porous space and thus sufficient adsorption capacity, the influence of different porogens and their combinations on the porous characteristics of the synthesized materials was studied. Table II presents the data from mercury intrusion porosimetry for 18 polymer samples obtained with the following porogens used in different ratios and combinations: CyOH, DoOH, PEG-400, and PEG-600.

As mentioned previously, the presence of a foreign solvent (1,4-dioxane) in the polymerization mixture leads to significant changes in the process of macroporous structure formation. Earlier, in the case of the synthesis of poly(GMA-*co*-EDMA) monoliths by photoinitiated free-radical polymerization, we demonstrated that the use of CyOH as the only porogen was absolutely sufficient to obtain a material with an average pore size of about 900–1000 nm.[28] In contrast, in the case of the preparation of poly(HPIEAA-*co*-GMA-*co*-EDMA), the use of CyOH as the only porogen led to the formation of a monolith with an average pore size of 30 nm (sample M1). Besides that, SEM data showed that the surface of this monolithic material had numerous defects [Fig. 2(a,b)].

PEG was chosen as a porogenic solvent because of its unique combination of hydrophilic and hydrophobic properties. Hydrophobic properties are defined by the presence of a polyoxyethylene chain, whereas hydrophilicity has to be related to OH groups located at the ends of the polymer chain.[29] In this study, PEGs with molecular masses of 400 and 600, which represent viscous liquids, were used. The application of PEG as the only porogenic solvent led to the formation of a final polymer product with very small pores (average pore size = 15 nm; sample M2).

TABLE II
Results of Mercury Intrusion Porosimetry

Monolith	Average pore size (nm)	Surface area (m^2/g)	Total porosity (%)
M1	30	78	62
M2	15	—	—
M3	15	—	—
M4	63	138	42
M5	1777	2	78
M6	20	155	40
M7	23	30	29
M8	169	21	47
M9	129	24	50
M10	72	85	48
M11	61	52	44
M12	59	58	52
M13	1890	14	52
M14	1347	5	64
M15	1574	4	41
M16	1160	44	53
M17	1607	4	61
M18	1452	5	71

All samples were obtained with an irradiation time of 30 min and an initiator concentration of 1%. The ratio of the porogens and monomers and porogen composition are shown in Table I.

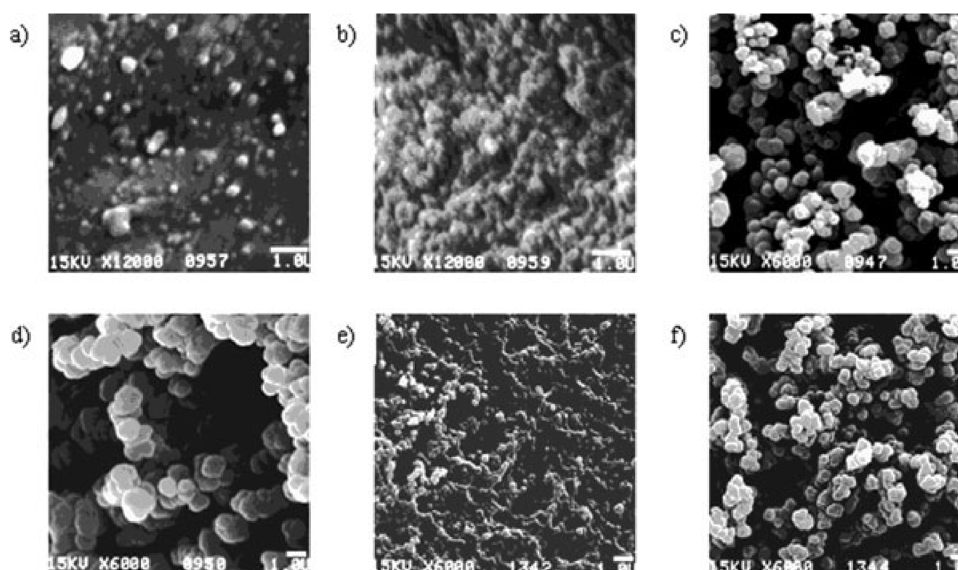

Figure 2 SEM images of the monoliths: (a) surface of sample M1, (b) internal surface (chip) of sample M1, (c) surface of sample M13, (d) internal surface (chip) of sample M13, (e) surface of sample M16, and (f) internal surface (chip) of sample M16.

The use of DoOH as a coporogen led to an increase in the average pore size in the case of both CyOH and PEGs. Moreover, as expected, a straight dependence between the amount of DoOH in the porogenic mixture and the pore size of the polymer product was observed. Obviously, the thermodynamic incompatibility of the porogenic solvents with the poly(HPIEAA-*co*-GMA-*co*-EDMA) copolymer increased in the following order:

$$1,4\text{-Dioxane} < \text{PEGs} < \text{CyOH} < \text{DoOH}$$

Thus, the addition of highly hydrophobic DoOH to the polymerization mixture led to decreasing thermodynamic incompatibility of the hydrophilic porogens with the synthesized polymer, and, consequently, the relative solvating ability of the monomers increased. Because the local concentration of the monomers inside the nuclei was bigger in the presence of DoOH versus CyOH or PEGs as the only porogens, it led to the formation of larger microglobular clusters and consequently larger space between them (macropores). However, the use of DoOH as the only porogen did not lead to the formation of a monolithic material at all.

Nevertheless, except for sample M5, the use of a porogen/monomer ratio of 60 : 40 led to the formation of a material with an average pore size in the range of 15–170 nm (samples M1–M4 and M6–M12), which is too low to consider these materials for further applications. The appropriate pore size was obtained only for samples M5 and M13–18, in which the PEG/DoOH mixture was used and the porogen/monomer ratio was 75 : 25. However, in the range of these copolymers, only two, M13 and M16 (Table II), had a satisfactory surface area (14 and 44 m^2/g, respectively), whereas for the other samples, it was found to be in the range of 2–5 m^2/g. This fact can be related to the pore structure specialties of the discussed monoliths. According to the data from intrusion mercury porosimetry, monoliths M13 and M16 had a bimodal porous structure (Fig. 3). In particular, sample M16 had mesopores of 15–30 nm and a partial volume equal to 15% of the total pore volume, whereas for monolith M13, the mesopore size and volume were found to be around 15 nm and 4.5%, respectively.

Other monolithic materials, namely M14, M15, M17, and M18, had no such kind of pore, and that, in turn, was reflected in their surface area. An increase in the porogen content in the polymerization mixture, in our case from 60 to 75%, seemed to be straightforwardly connected to early phase separation during polymerization and, consequently, was expressed in the acceleration of the process rate. In turn, the high reaction rate led to earlier nuclei precipitation and faster polymerization. As a result, the formation of a polymer product with no space between microglobules (no mesopores) took place. Moreover, an increase in the DoOH content was also connected to a reduction of the compatibility of the monomers with the porogenic mixture and thus led to early nuclei precipitation and faster polymerization, which in turn resulted in a monomodal polymer structure (Tables I and II).

For PEG-400 and PEG-600, the chain length insignificantly influences the average pore size of the synthesized material. A comparison of samples M10, M13, and M15 with samples M11, M17, and M18

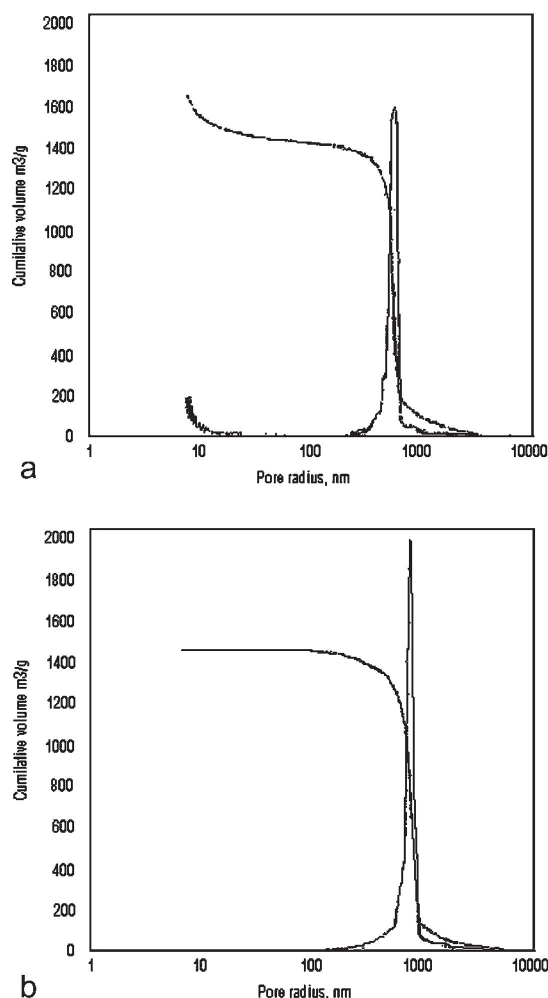

Figure 3 Pore size distribution of poly(HPIEAA-*co*-GMA-*co*-EDMA) determined by intrusion mercury porosimetry: (a) M16 and (b) M17. Conditions: initiator concentration = 1 mass %, irradiation time = 30 min, and porogen/monomer ratio = 75 : 25. For the porogen composition, see Table I.

leads to the conclusion that the average pore size really slightly decreases with the variation of the PEG chain length. Therefore, this tendency can be considered an additional factor allowing fine regulation of the material pore size.

Figure 2 presents micrographs of samples M1, M13, and M16 that well correlate with the data from mercury intrusion porosimetry. Obviously, polymer sample M13 demonstrated the best polymer morphology. Because monolith M13 appeared to be the most felicitous in terms of the pore size, surface area, and homogeneity of the polymer porous structure, this monolith was chosen for further investigation.

Kinetics of poly(HPIEAA-*co*-GMA-*co*-EDMA) synthesis

The kinetic curve of the copolymerization of HPIEAA, GMA, and EDMA is shown in Figure 4. Eighty-six percent of the monomers was converted

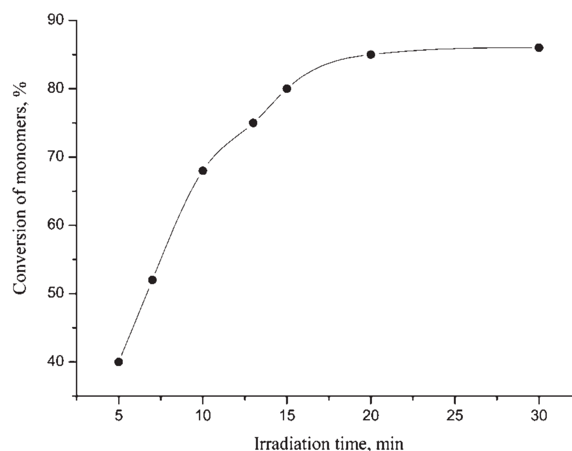

Figure 4 Kinetic curve of the polymerization process (sample M13). Conditions: initiator concentration = 1 mass % and porogen/monomer ratio = 75 : 25. For the porogen composition, see Table I.

into the polymer in 30 min. In the case of the photoinitiated polymerization of GMA with EDMA, the conversion of the monomers came to 96% in 20 min.[29] The reason for the observed difference is simple. Because both GMA and EDMA are aliphatic compounds, their UV absorption is less than that of the aromatic HPIEAA comonomer. Therefore, the dose used for terpolymer synthesis is diminished, and the polymerization is slower. Figure 5 demonstrates that the use of an initiator concentration of 0.8–1.0% leads to a maximum of the polymer yield.

Example of microanalysis on a constructed biochip

A model affinity pair of mouse IgG and goat anti-mouse IgG conjugated with Alexa Fluor 555 was

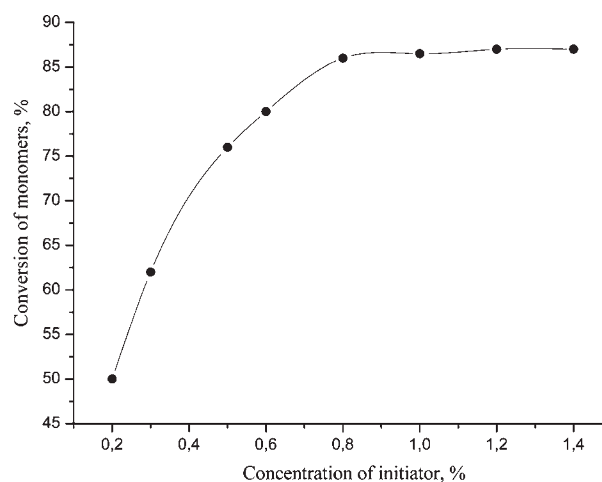

Figure 5 Dependence of the monomer conversion on the concentration of Darocur-1173 (sample M13). Conditions: irradiation time = 30 min and porogen/monomer ratio = 75 : 25. For the porogen composition, see Table I.

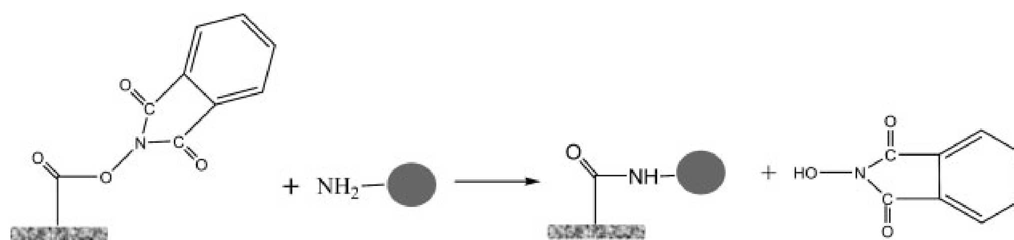

Figure 6 Scheme of the immobilization of an amino-bearing ligand on the surface of the poly(HPIEAA-*co*-GMA-*co*-EDMA) monolith.

used for biochip validation. A scheme of protein covalent immobilization on the surface of the poly (HPIEAA-*co*-GMA-*co*-EDMA) monolith is presented in Figure 6. For the purpose of determining the optimal immobilization conditions, mouse IgG was immobilized on the monolith's surface (sample M13 was used) at different pHs (7.5 and 9.5) and ligand concentrations (0.2–1.0 mg/mL). After a biospecific reaction of immobilized mouse IgG with dissolved goat anti-mouse IgG/Alexa Fluor 555 conjugate, it was demonstrated (Fig. 7) that the mean signal intensity was higher when ligand immobilization was carried out at pH 7.5. Additionally, experiments on the variation of the IgG concentration showed that immobilization of the protein from a solution with a concentration exceeding 0.2 mg/mL also resulted in a good signal intensity (Fig. 7).

The dependence of protein covalent attachment on the immobilization time is presented in Figure 8. The amount of protein bound to the solid matrix reached a maximum in 2 h of the immobilization process. After that time, the amount of immobilized ligand stayed constant. The amount of protein bound to the surface of the poly(HPIEAA-*co*-GMA-*co*-EDMA) monolith for 2 h was equal to 200 nmol of protein per gram of the monolithic support. A similar quantity of protein was bound to the surface of the poly(GMA-*co*-EDMA) material for 18 h under similar conditions. To prove that activated ester groups take part in the reaction with protein in the case of poly(HPIEAA-*co*-GMA-*co*-EDMA), but not epoxy groups belonging to the GMA comonomer, ligand immobilization was carried out on the surface of the poly(GMA-*co*-EDMA) monolith within the same time. Practically no protein attachment was observed in 2 h.

It is common in microarray-based analytical protocols to carry out a surface blocking procedure after affinity ligand immobilization. A BSA-containing buffer is widely used for this purpose. The application of such a mode of blocking is appropriate for the immobilization of big protein ligands because BSA can obscure small protein molecules. Because IgG with a molecular mass of approximately 150,000 Da was used as an immobilized ligand, the application of BSA as a blocking agent was acceptable.

Figure 9 presents an image of a fragment of a poly (HPIEAA-*co*-GMA-*co*-EDMA)-based biochip after affinity pair formation. It was determined that the detection limit for the developed test system based

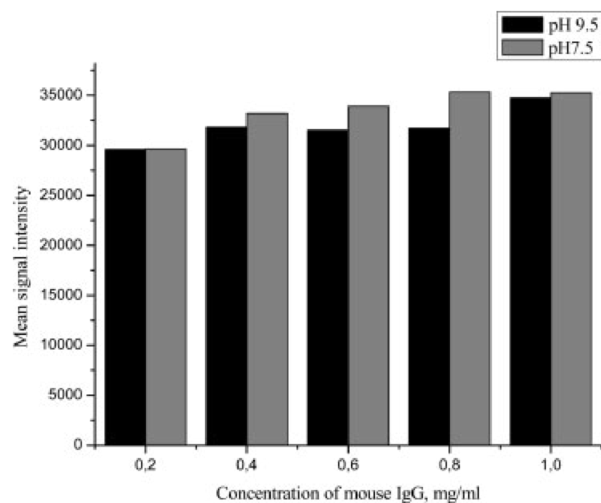

Figure 7 Dependence of the mean of signal intensity on the pH and concentration of the immobilized protein solution. Conditions: M13 and immobilization temperature = 37°C.

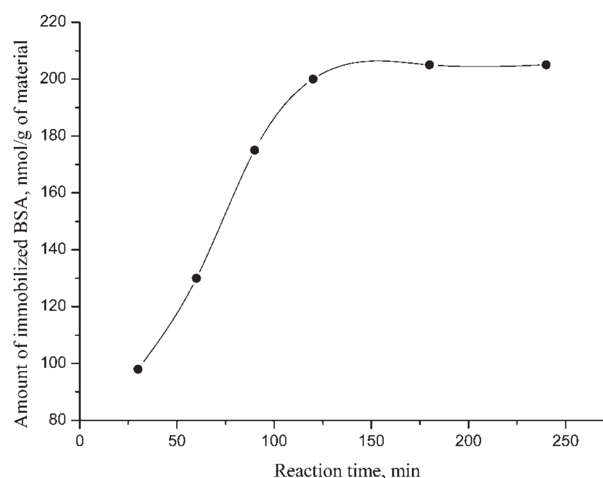

Figure 8 Kinetic curve of the protein immobilization on the monolithic surface. Conditions: M13, pH 7.5, and temperature = 37°C.

Figure 9 Image of the fragment of the biochip surface after microanalysis performance.

on the new macroporous support was equal to 20 ng/mL, which corresponded to 133 fmol/mL. The reproducibility of the results was estimated with intrafield and interfield coefficients of variation:

$$\text{Coefficient of variation} = \text{Standard deviation}/\text{Mean signal intensity} \quad (3)$$

The intrafield and interfield coefficients of variation were found to be equal to 17 and 30%, respectively.

CONCLUSIONS

In this study, the synthesis of new macroporous monoliths based on a terpolymer of HPIEAA, GMA, and EDMA was developed. The porous structure of the suggested material was optimized through the variation of the radiation time, concentration of the initiator, and nature and composition of the porogenic solvents.

This work represents a novel approach to the construction of monolithic three-dimensional protein microarrays. Despite research devoted to the development and thorough investigation of new monolithic copolymers with enhanced reactivity, a model example of microanalysis in a microarray format was realized, optimized, and discussed.

Obviously, this novel format of monolith application, namely, the three-dimensional protein microarray, has great potential for medical diagnostics, online analysis in biotechnology, and some other goals. The improvements in polymer synthesis will definitely lead to an increase in the quality of analysis on the microlevel and nanolevel.

The authors thank Aleš Štrancar (BIA Separations, Ljubljana, Slovenia) and E. N. Vlasova (Institute of Macromolecular Compounds, Russian Academy of Sciences) for intrusion porosimetry measurements and IR spectroscopy tests, respectively.

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