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Research Paper

Magnetic poly(glycidyl methacrylate) microspheres for protein capture

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The efficient isolation and concentration of protein antigens from complex biological samples is a critical step in several analytical methods, such as mass spectrometry, flow cytometry and immunochemistry. These techniques take advantage of magnetic microspheres as immunosorbents. The focus of this study was on the development of new superparamagnetic polymer microspheres for the specific isolation of the tumor suppressor protein p53. Monodisperse macroporous poly(glycidyl methacrylate) (PGMA) microspheres measuring approximately 5 μm and containing carboxyl groups were prepared by multistep swelling polymerization of glycidyl methacrylate (GMA), 2-[(methoxycarbonyl)methoxy]ethyl methacrylate (MCMEMA) and ethylene dimethylacrylate (EDMA) as a crosslinker in the presence of cyclohexyl acetate as a porogen. To render the microspheres magnetic, iron oxide was precipitated within their pores; the Fe content in the particles received ~18 wt%. Nonspecific interactions between the magnetic particles and biological media were minimized by coating the microspheres with poly(ethylene glycol) (PEG) terminated by carboxyl groups. The carboxyl groups of the magnetic PGMA microspheres were conjugated with primary amino groups of mouse monoclonal DO-1 antibody using conventional carbodiimide chemistry. The efficiency of protein p53 capture and the degree of nonspecific adsorption on neat and PEG-coated magnetic microspheres were determined by western blot analysis.

Introduction

Magnetic immunosorbents are receiving increasing attention in the biosciences, including nanomedicine, biotechnology, microbiology and molecular biology [1]. Easy manipulation and high binding capacity are the main advantages of magnetic microspheres, which find various applications in cell separation [2,3] and the analysis of proteins [4], nucleic acids [5,6] and enzymes [7]. The presence of suitable functional groups on the particle surface allows for the binding of specific biomolecules, such as antibodies [8], enzymes [9] or polysaccharides [10]. Not only the amount of attached biomolecules but also their corresponding function, for example, antibody or enzyme activity, and nonspecific interactions strongly depend on the characteristics of their reactive groups, as well as their quantity and surface distribution [11]. To ensure a sufficient difference between the nonspecific and specific binding of analytes, magnetic immunosorbents must suppress the nonspecific adsorption of cells, proteins and other biomolecules. The formation of biocompatible surfaces resisting protein and platelet adhesion is usually achieved by applying various coatings based mainly on poly(ethylene glycol) (PEG) [12], dextran [13], albumin [8,14] and other natural or synthetic polymers. Recently, zwitterionic polymers based on phosphorylcholine [15], sulfobetaine [16] and carboxybetaine [17] containing anionic and cationic end-groups have become increasingly attractive for their antifouling properties.

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Magnetic immunosorbents are typically micrometer-sized, monodisperse, spherical polystyrene (PS) particles whose surfaces are frequently modified to increase their hydrophilicity and biocompatibility. The advantage of the newly developed magnetic poly(glycidyl methacrylate) (PGMA) over commercial PS microspheres consists in the absence of aromatic moieties and thus minimal autofluorescence, which makes the particles highly suitable for analytical methods based on fluorescence measurements. The properties demanded of magnetic particles are analogous to those demanded of non-magnetic ones, including an appropriate size, monodispersity and stability (no aggregation) in various biological media. Moreover, magnetization is necessary to ensure a quick response to an applied magnetic field and an efficient separation process. Ideally, the particles should be superparamagnetic to be redispersible in media when the magnetic field is removed.

Magnetic PGMA microspheres can be prepared by a number of heterogeneous polymerization techniques carried out in the presence of magnetic nanoparticles or by the precipitation of iron salts in particle pores [18]. The particles produced by these approaches vary in size, polydispersity, and magnetic filler content, as well as in their intended applications. Magnetic PGMA microspheres (>40 μm) suitable for magnetic separation were prepared by suspension polymerization in the presence of manganese perovskite [19]. Analogous submicrometer particles were produced by the emulsion [20] or miniemulsion polymerization [21]. Another convenient method for preparing magnetic PGMA microspheres is dispersion polymerization, which is easy to perform and provides particles of uniform size [22,23]. However, the addition of another agent (e.g., crosslinker) during dispersion polymerization affects the nucleation mechanism and broadens the particle size distribution [24]. In contrast, multistep swelling polymerization, pioneered by Ugelstad et al. [25], is complex and elaborate but provides robust monodisperse microspheres that are resistant to aggregation. Moreover, the method can produce porous microspheres in which magnetic compounds are prospectively formed. The technique is used to produce magnetic DynaBeads, which are considered the gold standard in the field of protein and cell separation because they are tailored to specific biological applications [26]. The microspheres are sold in several sizes (typically 2.8 and 4.5 μm) and exhibit a range of functionalities, such as amino, carboxyl or tosyl groups, streptavidin, protein A and G and a variety of antibodies.

The aim of this study was to confirm the applicability of innovative magnetic PGMA microspheres and their PEGylated counterparts with immobilized DO-1 antibody exhibiting high affinity towards the p53 protein for immunoprecipitation from the cell lysate. PEGylation under 'cloud-point' conditions is generally used on planar surfaces to produce densely coated functional substrates [27–29]; however, the process is not commonly carried out on spheres. Control over the concentration of the functionalities allows for highly efficient protein capture as well as minimal nonspecific adsorption to be achieved.

Tumor suppressor protein p53, which arrests the G1 phase of the cell cycle in response to DNA damage [30,31], plays a key role in the regulation of cell proliferation and apoptosis [32]. Moreover, as a transcription factor, the protein controls gene expression involved in cellular defense against malignant transformations [32]. P53 is the most frequently altered gene in human cancers [33,34], and the presence of a P53 mutation is generally associated with poor patient prognosis and may lead to poor response to conventional therapy. However, the p53 malfunction in cancer can also be caused by the alteration of the protein's regulatory proteins (e.g., MDM2, MDMX) by viral proteins, such as E6, E1A and T-antigen, or by altered kinase signalization [35–37]. Analyses of p53 expression level, DNA binding capacity, posttranslational modifications and interaction partners are therefore as important as the detection of p53 mutations [38–40]. New approaches that could examine various p53 alterations in complex biological samples depending on the specific isolation and concentration of the protein are thus needed.

Experimental

Materials

Styrene (Synthos; Kralupy, Czech Republic), glycidyl methacrylate (GMA; Fluka; Buchs, Switzerland) and ethylene dimethacrylate (EDMA; Röhm; Darmstadt, Germany) were vacuum-distilled. 2-[(Methoxycarbonyl)methoxy]ethyl methacrylate (MCMEMA) was prepared according to a procedure described in a previous report [41]. 2,2′-Azobis(2,3,3-trimethylbutanonitrile) (ABTB) was obtained from 3,3-dimethylbutan-2-one, hydrazine, hydrogen cyanide and bromine [42]. Cyclohexyl acetate was obtained from cyclohexanol and acetic anhydride. Methocel 90 HG [(hydroxypropyl)methyl cellulose], 3-morpholino-2-hydroxypropanesulfonic acid (MOPSO) and succinic anhydride (SA) were products of Fluka. Sodium dodecyl sulfate (SDS), dibutyl phthalate (DBP), $FeCl_2 \cdot 4H_2O$, *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDC), *N*-hydroxysulfosuccinimide sodium salt (sulfo-NHS), *N*,*N*-diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA) and triisopropylsilane (TIPS) were obtained from Sigma–Aldrich (St. Louis, USA). NH_4OH and other common chemicals were obtained from Lachema (Neratovice, Czech Republic). α,ω-Bis-carboxy poly(ethylene glycol) (α,ω-PEG-COOH; $M_w = 2000$) was purchased from Iris Biotech (Marktredwitz, Germany), and α-methoxy-ω-amino poly(ethylene glycol) (CH_3O-PEG-NH_2; $M_w = 5079$) and α-amino-ω-*t*-Boc-amino poly(ethylene glycol) (H_2N-PEG-NH-*t*-Boc; $M_w = 4847$) were purchased from Rapp Polymere (Tübingen, Germany). 2-(N-morpholino)ethanesulfonic acid (MES), ethanolamine (EA) and 2-mercaptoethanol were purchased from Sigma–Aldrich, and lithium dodecyl sulfate (LDS) was obtained from Life Technologies (Carlsbad, USA). Ultrapure Q-water was ultrafiltered on a Milli-Q Gradient A10 system (Millipore, Molsheim, France). DO-1 and CHIP 11.1 mouse monoclonal antibodies were obtained from Moravian-Biotechnology (Brno, Czech Republic), and RAM Px rabbit polyclonal antibody was obtained from Dako (Glostrup, Denmark).

Synthesis of monodisperse polystyrene (PS) seeds

PS seeds were obtained by the emulsifier-free emulsion polymerization of styrene in a 150-ml reaction vessel equipped with an anchor-type stirrer. Briefly, sodium persulfate (44 mg) and sodium carbonate (39 mg) were dissolved in water (90 ml) to form the aqueous phase. The aqueous phase was mixed with styrene (10 g), the emulsion was stirred (300 rpm), and the temperature was increased to 80°C to initiate the polymerization, which then proceeded for 20 hours under a nitrogen atmosphere. The resulting latex was separated by centrifugation and thoroughly washed with a 0.25% aqueous SDS solution.