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Amine-functionalization of glycidyl methacrylate-containing emulsion-templated porous polymers and immobilization of proteinase K for biocatalysis☆

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ABSTRACT

Glycidyl methacrylate (GMA) emulsion-templated porous polymers (polyHIPEs) were prepared by thermal and photopolymerisation and derivatised with morpholine, tris(2-aminoethylamine) and a bisamino-PEG homobifunctional molecule. The extent of the functionalization reactions was investigated by a range of qualitative and quantitative techniques (FTIR, CHN analysis, titration, XPS, HR-MAS NMR spectroscopy, ninhydrin assay and Fmoc number determination) and was found to be excellent for small molecule amines (up to 89% conversion) but low for the reaction with PEG (2% conversion). This was ascribed to the high exclusion volume of the PEG chains in solution. Proteinase K (Pro K) was subsequently immobilized covalently onto the GMA polyHIPE material, both directly via reaction with surface epoxy groups and indirectly by activation of the pendent amine groups of PEGylated polyHIPE with glutaraldehyde then reductive amination with the enzyme. The activity of the supported enzymes was determined by a continuous electrochemical assay involving the hydrolysis of N-acetyl-L-tyrosine ethyl ester. The directly immobilized Pro K was found to have an activity of only 3.6 U/g whereas the activity of the enzyme immobilized via the PEG linker was much higher (up to 78 U/g).

1. Introduction

Functional polymers can be prepared by the homopolymerization, copolymerization or grafting of a reactive monomer [1–3]. Poly(glycidyl methacrylate) (GMA) is an important functional polymer due to the ability of its epoxy group to react with a range of nucleophiles [1]. This has led to the preparation of porous GMA polymers for use as bioreactors and for protein separation [4–6]. Highly porous monolithic polymers can be prepared by emulsion-templating, in which a high internal phase emulsion (HIPE) is used to create a fully interconnected network of pores in the approximate size range 1–100 μm [7–12]. Such polyHIPE materials have advantages over other monolithic porous materials including a high (95%+) and fully interconnected porosity and the ability to prepare large monoliths [13–15]. Recently, GMA-based polyHIPE materials have been prepared by thermal or photochemical polymerisation of GMA as well as the grafting of GMA from a polyHIPE surface [16–21]. These materials have been observed to be capable of functionalization with nucleophiles and have been used for protein separation [18,22].

Enzymes are stereo- and regio-specific catalysts that react under relatively mild conditions [23] and can be used on an industrial scale [24]. For example, lipases have been used in the synthesis of a range of pharmaceutical intermediates and also bulk chemicals [25–30]. Proteases, whose natural function is the hydrolysis of amide bonds, can catalyse the formation of peptide bonds via either thermodynamic or kinetic control [23,31,32], and are being researched intensely for the production of di- and oligo-peptides [32–35]. Protease-catalysed peptide synthesis has several advantages over solid phase peptide synthesis methods, including mild reaction conditions and increased enantioselectivity [23].

Immobilization of enzymes on a substrate improves ease of handling, increases stability and facilitates recycling thus reducing cost of enzyme reuse [36–39]. Recently, polyHIPEs have been investigated as a potential material for the covalent immobilization of lipases [40–43]. Despite a relatively low surface area (ca. 5 m^2/g) and low enzyme loading in comparison with a commercially

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available product, the lipase *Candida Antarctica* Lipase B (Cal-B) immobilized on polyHIPE was shown to have a higher activity and could be re-used several times without any reduction in activity, which was attributed to a greater accessibility of the enzyme to the substrate [43].

This work concerns the preparation of functional GMA polyHIPEs for the covalent immobilization of proteases. Unlike lipases, proteases are usually immobilized on hydrophilic materials and so an alternative support material to that used in our previous work [43] is required. To test suitability for enzyme immobilization, the epoxy group of GMA polyHIPE was functionalized with a range of amine nucleophiles. One of these, a bis-amino terminated polyethylene glycol (PEG), was immobilized onto the polyHIPE as a means to space the enzyme from the polymer surface. Spacer groups can increase the stability of an enzyme and retain its activity on immobilization, in comparison to direct immobilization [37,44–47]. PEG was chosen because of its ability to prevent the non-specific adhesion of proteins, due to the high exclusion volume of PEG chains in aqueous solution [48], and to also provide a hydrophilic surface for the immobilized protease. PEG linkers have previously been used for the covalent attachment of enzymes to solid supports [45]. Proteinase K (Pro K) from *Tritirachium album* was subsequently immobilized onto GMA-based polyHIPEs, both directly and via the PEG spacer. The activity of the immobilized Pro K was monitored with a continuous electrochemical assay, monitoring the hydrolysis of N-acetyl-L-tyrosine ethyl ester monohydrate.

2. Experimental section

2.1. Materials

O,O'-Bis(3-aminopropyl)polyethylene glycol (Sigma–Aldrich; $M_n \sim 1500$), tetrahydrofuran (Fisher Scientific, laboratory reagent grade), buffer tablets pH 9.2 (borate) (Fisher Scientific), fluorescein 5(6)-isothiocyanate (Sigma; ~90%), morpholine (Sigma–Aldrich; ≥99%), tris(2-aminoethyl)amine (Aldrich; 96%), 9-fluorenylmethyl chloroformate (Aldrich, 97%), piperidine (Sigma–Aldrich, 99%), N,N-dimethylformamide (Sigma–Aldrich, ≥99.8%), dichloromethane (Fisher Scientific, analytical grade), chloroform-d (Sigma–Aldrich, 99.8 atom % D), ethanol (Fisher Scientific, > 99% (GLC)), N,N-diisopropylethylamine (Sigma–Aldrich, 99.5%), methanol (Fisher Scientific, HPLC grade), ninhydrin (Sigma, ≥99%), hydrochloric acid (Fisher Scientific, Laboratory grade (~36%)), glycidyl methacrylate (GMA; Fluka, 97%), ethyleneglycol dimethacrylate (EGDMA; Aldrich, 98%), 2-ethylhexyl acrylate (EHA; Aldrich, 98%), trimethylolpropane triacrylate (TMPTA; Aldrich, technical grade), Hypermer B246 (triblock copolymer of poly(12-hydroxystearic acid) and poly(ethylene glycol) with an HLB number of 6) (Univar Ltd.), Synperonic PEL 121 (triblock copolymer of poly(propylene oxide) and poly(ethylene oxide), with an HLB number of 0.5) (Croda), diphenyl(2,4,6-trimethylbenzoyl)phosphine oxide/2-hydroxy–2–methylpropiophenone, blend (Aldrich), sodium phosphate monobasic (Sigma–Aldrich, ReagentPlus®, ≥99.0%), sodium hydroxide (Sigma–Aldrich, reagent grade, ≥98%), glutaraldehyde (Sigma–Aldrich, 50 wt. % in H_2O), sodium cyanoborohydride (Fluka, purum, ≥95.0%), proteinase K from *T. album* (Sigma, lyophilized powder, ≥30 units/mg protein), bovine serum albumin (Bio-rad, 2.15 mg/ml standard solution in H_2O), Bradford Reagent (Bio-rad, concentrated solution, contains Coomassie brilliant blue, methanol, and phosphoric acid) and N-acetyl-L-tyrosine ethyl ester monohydrate (Aldrich, 99%) were all used as supplied.

Phosphate buffers were prepared prior to use with sodium phosphate monobasic. Concentrations of sodium phosphate monobasic buffer and pH were adjusted accordingly. Buffers were stored at 4 °C prior to use and were discarded after one month. Cellulose acetate syringe filters (0.45 μm porosity, 13 mm diameter) were obtained from Cronus®. Semi-micro disposable polystyrene cuvettes of 4.0 mL capacity and 10 mm path length were purchased from Fisher Scientific.

2.2. Thermally polymerised GMA-based polyHIPE preparation

The GMA-based thermally polymerised polyHIPEs were prepared with a nominal porosity of 80%, based on the aqueous phase content. An oil phase consisting of glycidyl methacrylate (14.51 g, 0.1 mol), ethylene glycol dimethacrylate (6.76 g, 34 mmol), and surfactant Synperonic PEL 121 (4.28 g, 20% w/w of oil phase) was added to a 250 mL three-necked round bottomed flask. Thus the mole percentages of the monomers are: EGDMA 25%; GMA 75%. The oil phase was then stirred continually at 350 rpm using a D-shaped PTFE paddle connected to an overhead stirrer. An aqueous phase consisting of 80 mL of deionised water, water soluble initiator potassium persulfate (0.2% w/v of aqueous phase), and calcium chloride hexahydrate (2% w/v of aqueous phase) was added over a period of 30 min, then the HIPE was left to stir for an additional 30 min. The HIPE was then transferred to a polycarbonate centrifuge tube, which was then placed in an oven at 60 °C for 24 h. The resulting monolith was recovered from the tube then extracted in a Soxhlet apparatus with deionised water for 24 h, then with ethanol for 24 h, and dried *in vacuo* at 55 °C for a minimum of 24 h.

2.3. Photopolymerised GMA-based polyHIPE preparation

The formulation used to prepare GMA-functionalized polyHIPEs was based on that employed previously [49] and is a modification of a commonly employed mixture for the production of polyHIPEs by photopolymerisation. The GMA-based photopolymerised polyHIPEs were prepared with a nominal porosity of 95%, based on the aqueous phase content. Briefly, a w/o HIPE was obtained from the addition of an aqueous phase to an oil phase containing a surfactant under the application of stirring. The oil phase consisting of glycidyl methacrylate (GMA; 0.73 g, 5.1 mmol), 2-ethylhexyl acrylate (EHA; 3.66 g, 19.9 mmol), isobornyl acrylate (IBOA; 0.87 g, 4.2 mmol), trimethylolpropane triacrylate (TMPTA; 1.41 g, 4.8 mmol), surfactant Hypermer B246 (0.2 g, 3% w/w of oil phase) and photoinitiator diphenyl(2,4,6-trimethylbenzoyl)phosphine oxide/2-hydroxy–2–methylpropiophenone, blend (0.78 g, 10% v/v of monomer phase) was added to a 250 mL two-necked round bottomed flask. Thus the mole percentages of the various monomers are: GMA 15 mol%; EHA 59 mol%; IBOA 12 mol%; TMPTA 14 mol%. Monomer, crosslinker, photoinitiator and surfactant were mixed in the dark. The oil phase was then stirred continually in the dark at 350 rpm using a D-shaped PTFE paddle connected to an overhead stirrer. An aqueous phase consisting of 63 mL of deionised water was added dropwise to the oil phase over a period of 10 min, and then the HIPE was left to stir for an additional 10 min to produce a homogenous emulsion. The HIPE was then placed between two glass plates within a PTFE square ring (50 × 50 × 5 mm). This was then exposed to the UV lamp three times on each side at 3.5 m/min (conveyor belt speed) at 100% lamp intensity. The resulting elastomeric monolith was recovered from between the glass plates and washed in acetone (5 × 500 mL) and then dried *in vacuo* at 55 °C for a minimum of 24 h.

2.4. Functionalisation of GMA-based PolyHIPE materials

Photopolymerized GMA polyHIPEs were reacted with two small molecule amines, morpholine and tris(2-aminoethyl)amine, to assess the availability of the epoxy groups for reaction (Scheme 1).