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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).

Prior to reaction, the materials were ground into a powder using a mortar and pestle to ensure homogeneous functionalization.

2.5. Morpholine and tris(2-aminoethyl)amine (trisamine)

2.5.1. Method 1

1.0 g (0.77 mmol of epoxy groups) powdered photopolymerised GMA-based polyHIPE was added to 80 mL of a 12.5% v/v (115 mmol) morpholine (or (67 mmol) trisamine)/THF solution at 0 °C and stirred for 10 min. The mixture was then stirred for 2 h at room temperature. The polyHIPE was washed with THF (2 × 50 mL), water (2 × 50 mL), ethanol (2 × 10 mL) and diethyl ether (10 mL) and dried *in vacuo* at 55 °C for 24 h.

2.5.2. Method 2

1.0 g (0.77 mmol of epoxy groups) powdered photopolymerised GMA-based polyHIPE was added to 80 mL of a 12.5% v/v (115 mmol) morpholine (or (67 mmol) trisamine)/THF solution at room temperature and stirred for 10 min. The mixture was then stirred for 24 h at reflux. The polyHIPE was washed with THF (2 × 50 mL), water (2 × 50 mL), ethanol (2 × 10 mL) and diethyl ether (10 mL) and dried *in vacuo* at 55 °C for 24 h.

2.6. O,O′-bis-(3-aminopropyl) polyethylene glycol

Thermally polymerised GMA polyHIPEs were reacted with *O,O′*-bis(3-aminopropyl)polyethylene glycol. 200 mg (0.15 mmol of epoxy groups) of GMA-based polyHIPE was added to 40 mL of a solution of 1.5 g (1 mmol) of *O,O′*-bis(3-aminopropyl)polyethylene glycol in tetrahydrofuran (THF) and stirred at room temperature for 24 h. The polyHIPE material was then washed with THF (3 × 50 mL) followed by ultra high purity water (6 × 50 mL). The polyHIPE material was then freeze-dried for 24 h.

2.7. Quantification of amine loading

2.7.1. O,O′-Bis(3-aminopropyl)polyethylene glycol—determination of amine loading from Fmoc number determination

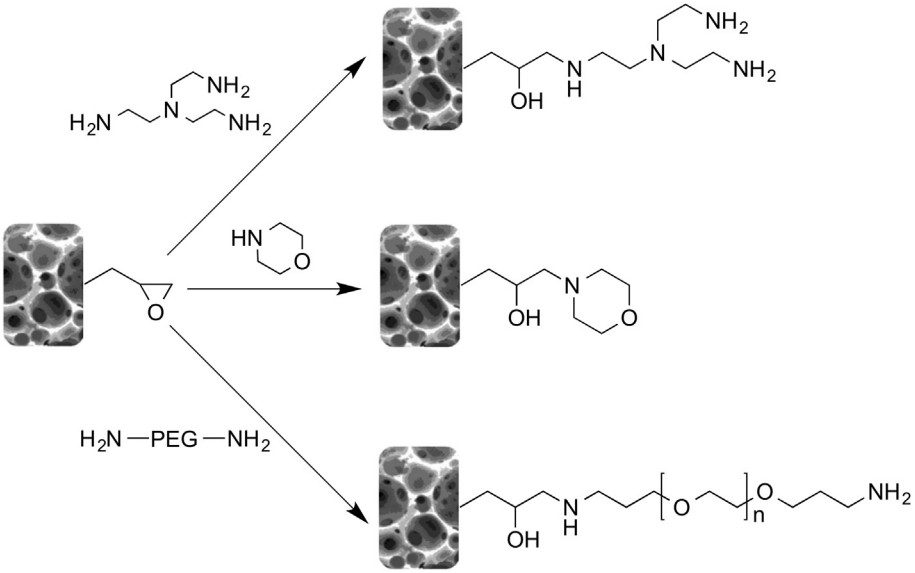
The determination of free $-NH_2$ groups followed the method by Badyal et al. (see Scheme 2) [50]. *O,O′*-Bis(3-aminopropyl)polyethylene glycol functionalized GMA polyHIPE (30 mg, 0.144 mmol of amine groups assuming complete functionalization with PEG), 9-fluorenylmethyl chloroformate (Fmoc-Cl) (75 mg, 0.3 mmol), N,N-diisopropylethylamine (DIPEA) (50 μL, 0.29 mmol) and dichloromethane (1 mL) were loaded into a 10 mL glass vial fitted with a screw cap. The mixture was shaken on a roller shaker for 1.5 h. The polyHIPE was then filtered under reduced pressure and washed with dichloromethane (10 × 5 mL) and dried *in vacuo* at 50 °C for 24 h.

Deprotection of the Fmoc-amine groups involved the addition of 10 mg (0.048 mmol of Fmoc protected amine groups assuming complete functionalization with PEG) of the dried Fmoc-protected polyHIPE to a 5 mL volumetric flask, followed by 400 μL of a 20% (0.81 mmol) piperidine/N,N-dimethylformamide (DMF) solution and shaking for 30 min at room temperature. Methanol was then added to the volumetric flask to obtain a 5 mL solution. A portion of the solution (200 μl) was then removed and diluted 25 times with methanol. Absorbance readings of the diluted solution were recorded on a UV-Vis spectrometer at 301 nm (related to the piperdine adduct deprotection product) and 322 nm (backround reading). The Fmoc loading was calculated from the Beer–Lambert law (see equation (1)). The Fmoc procedure was performed in duplicate in order to obtain an average Fmoc number. A normalised Fmoc value is recorded from the value obtained from taking into consideration the molecular weight of the Fmoc group (7800 is the molar extinction coefficient of the Fmoc-piperidine adduct, see Scheme 2).

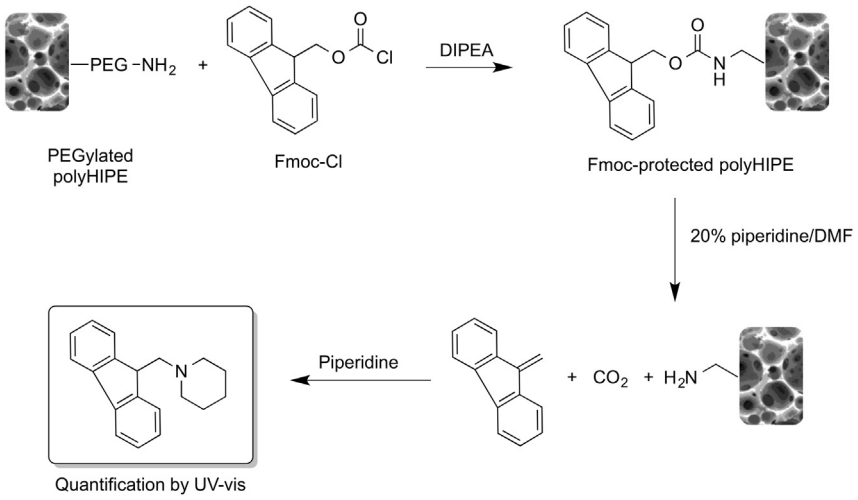
$$\text{Loading in mmolg}^{-1} = \left(\frac{\text{Absorbance}}{7800}\right) \times \text{Dilution} \times \left(\frac{\text{Flask volume(mL)}}{\text{Wt. of sample(g)}}\right) \quad (1)$$

2.7.2. Determination of morpholine loading

Dried morpholine functionalized polyHIPE material (0.2 g) was added to 5 mL of 0.5 M hydrochloric acid solution and left to stand in the solution overnight. The mixture was then filtered and 3 mL of this filtrate was then titrated with a dilute (0.05 M) NaOH solution [51].



Scheme 1. Functionalization of GMA-based polyHIPEs with amines.



Scheme 2. Quantification of $-NH_2$ groups on PEGylated GMA polyHIPE by reaction with 9-fluorenylmethyl chloroformate (Fmoc-Cl) followed by removal of Fmoc with 20% piperidine/DMF solution to obtain the piperidine adduct, which absorbs at $\lambda = 301$ nm (UV). DIPEA = N,N-diisopropylethylamine.

2.7.3. Ninhydrin (Kaiser) test

The following qualitative test for the presence of primary amine groups with ninhydrin (the so-called Kaiser test [52]), was adapted from Coin et al. [53]. 50 mg of powdered *O,O′*-bis(3-aminopropyl) polyethylene glycol functionalised polyHIPE was added to 1.5 mL of 1.0 mol dm^{-3} ninhydrin solution in ethanol. This was stirred at room temperature for 5 min and then heated at 70 °C for 15 min. The polyHIPE was then washed with 5 × 15 mL aliquots of ethanol and the polyHIPE was dried *in vacuo* at 55 °C for 24 h. Unreacted GMA polyHIPE was used as a control for this ninhydrin reaction.

2.7.4. Fluorescein 5(6) – isothiocyanate (FITC)

50 mg of *O,O′*-bis(3-aminopropyl)polyethylene glycol functionalised polyHIPE was added to 10 mL of 26 μmol dm^{-3} fluorescein 5(6)-isothiocyanate solution in pH 9.2 borate buffer and was stirred at room temperature for 24 h in a 20 mL glass vial covered with aluminium foil. The material was then washed with pH 9.2 borate buffer (2 × 50 mL) and ethanol (2 × 10 mL) and then dried *in vacuo* at 55 °C for 24 h. Unfunctionalized powdered polyHIPE was used as a control material for analysis purposes. After functionalization with FITC the polyHIPEs were placed under a UV lamp of wavelength 254 nm for visualisation.

2.8. Immobilization of proteinase K onto GMA-based emulsion-templated porous polymers

Proteinase K (Pro K) was immobilized by direct reaction between lysine residues on the enzyme surface and the epoxy groups on GMA polyHIPE. Powdered polyHIPE was added to a 1 mg/mL solution of Pro K from *T. album* in 20 mM pH 8.0 phosphate buffer (10 mg of polyHIPE per 1 mL of Pro K solution) and stirred at room temperature for 24 h. PolyHIPE material was washed with 20 mM pH 8.0 phosphate buffer (5 × 10 mL) and 20 mM pH 7.0 phosphate buffer (5 × 10 mL), then stored at 4 °C in pH 7.0 phosphate buffer until further use.

Pro K was also immobilized on PEGylated polyHIPE materials. The amino terminal group of the PEG spacer was reacted with glutaraldehyde prior to immobilization of Pro K (Scheme 3) [54–56]. Powdered PEGylated polyHIPE material (400 mg) was added to 20 mL of a 10% glutaraldehyde solution and stirred at 30 °C for 3 h. Powdered glutaraldehyde-functionalized polyHIPE was then washed with 20 mM pH 8.0 phosphate buffer (5 × 10 mL) and was then added to a 1 mg/mL solution of Pro K in 20 mM pH 8.0 phosphate buffer (10 mg of polyHIPE per 1 mL of Pro K solution) and stirred at 4 °C for 48 h. Reduction of the imine groups followed the protocol from Hermanson [54], whereby 10 μL of 5M sodium cyanoborohydride in 20 mM pH 8.0 phosphate buffer was added to the mixture and stirred for 2 h at room temperature. PolyHIPE material was then washed with 20 mM pH 8.0 phosphate buffer (5 × 10 mL) and 20 mM pH 7.0 phosphate buffer (5 × 10 mL). PolyHIPE was then stored at 4 °C in pH 7.0 phosphate buffer until further use.

2.9. Pro K activity continuous titrimetric assay

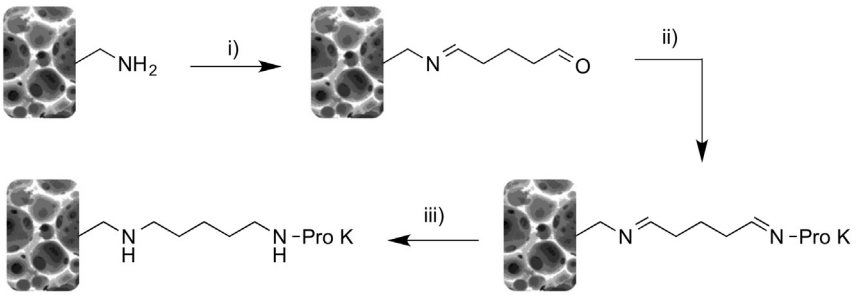
The titrimetric assay described by Ebeling et al. was followed to determine the activity of Pro K [57]. This involves the hydrolysis of N-acetyl-L-tyrosine ethyl ester monohydrate (ATEE) at pH 9.0 and 30 °C. An autotitrator consisting of an electrode, pH meter, a motor-driven 25 mL volume burette, and a magnetically stirred reaction vessel with water jacket was used for the electrochemical pH-stat assay. An external water bath and piston pump were used to control the temperature of the reaction vessel. The following reagents were prepared: 50% (w/w) methanol solution in ultra-high purity (UHP) water, 50 mM ATEE in 50% (w/w) methanol solution, 500 mM calcium chloride solution in UHP water, 60 mM sodium hydroxide solution in UHP water. To a stirred thermostatted reaction vessel powdered Pro K immobilized polyHIPE material (50–600 mg), 12.0 mL of UHP water and 4.0 mL of calcium chloride solution was added and incubated at 30 °C for 20 min. This was then followed by the addition of 4.0 mL of 50 mM ATEE in 50% (w/w) methanol solution in UHP water. The pH stat was then pre-dosed with 60 mM sodium hydroxide until a pH 9.0 was reached prior to the start of the assay. Equation (2) was used for the calculation of the activity (in units) of the immobilized enzyme per g of polyHIPE material.

$$\text{Units/gpolyHIPE} = \left(\frac{(\text{Molarity of NaOH}) \times (\text{Vol. NaOH}) \times (1000)}{(\text{gpolyHIPE}) \times (T)}\right) \quad (2)$$

where, one unit (U) is defined as the hydrolysis of 1.0 μmol of ATEE per minute at pH 9.0 at 30 °C, T is the time taken for the assay (in min.).

2.10. Instrumentation

Powdered polyHIPE samples were prepared by grinding by hand using a mortar and pestle. If necessary, samples were cooled using liquid nitrogen to aid grinding. ATR-FTIR spectra were acquired on a



Scheme 3. Immobilization of Pro K onto PEGylated polyHIPE material: i) activation of the PEGylated polyHIPE with glutaraldehyde; ii) immobilization of Pro K onto polyHIPE; iii) reduction of imine bonds with sodium cyanoborohydride.

Nicolet Nexus FTIR spectrometer with STI golden gate. Powdered samples were used. 16 scans were taken for both background and sample with a resolution of 4 cm^{-1}. The spectra were baseline-corrected. XPS of powdered polyHIPE samples was obtained using a Kratos AXIS ULTRA XPS used in FAT (fixed analyser transmission) mode using mono-chromated Al kα X-ray source (1486.6 eV) operated at 15 mA emission and 12 kV anode potential – 180 W. This was 'charge corrected' to C 1s peak at 285 eV. XPS energy range was calibrated using copper 2p/gold 4f and silver 3d peaks. A wide survey scan and high resolution scan was performed on each sample. The wide angle scan was over all energy ranges that allowed for detection of all elemental photoelectron peaks (i.e. 1400–0 eV, except hydrogen and helium). All samples were placed under vacuum at 50 °C for 24 h prior to XPS analysis. Casa XPS™ software was used to view the data obtained [58].

Solution state NMR spectra were acquired on a Varian 400 spectrometer with $CDCl_3$ as solvent. High Resolution Magic Angle Spinning (HR-MAS) NMR spectroscopy was carried out on a Varian 500 spectrometer with $CDCl_3$ as solvent, with the use of a Varian Nanoprobe. A few mg of dried powdered polyHIPE was added to the sample tube, prior to the addition of 40 μL of $CDCl_3$. The polyHIPE was allowed to swell for 30 min prior to analysis. Samples were spun at ~54° at a spin rate of 1000 Hz to reduce signal broadening within the ^1H NMR spectrum. GMA polyHIPE was also analysed via ^1H HR-MAS NMR spectroscopy as a comparison to the PEGylated material. Analysis of NMR spectra was undertaken using the software Mestrenova© [59].

A Varian Cary 100 Spectrophotometer was used for the calculation of Fmoc number and determination of FITC absorbance. CHN elemental analysis was carried out using an Exeter Analytical Inc. CE-440 Elemental Analysis. A Radiometer TIM 856 pH-Stat autotitrator was used for Pro K continuous titrimetric assay.

3. Results and discussion

3.1. Amine functionalization of GMA-based PolyHIPEs

Tris(2-aminoethyl)amine (trisamine) was reacted with GMA-functionalized polyHIPE due to its high nitrogen content that would enable accurate determination of loading via elemental analysis. Morpholine was also used since, in addition to elemental analysis, its loading can be determined by back-titration. Both trisamine and morpholine have been used previously to assess the level of functionalization of polyHIPEs post-polymerisation [9,60–62].

FTIR analysis of GMA polyHIPE showed a distinctive (meth) acrylate ester peak at 1724 cm^{-1} and epoxy peaks at 906 and 845 cm^{-1} (see Fig. 1, lower spectrum). Functionalization with trisamine for 2 h at room temperature caused a noticeable reduction in the intensity of the latter peaks due to ring opening of the epoxy group of GMA, however residual epoxy groups clearly remained (see Fig. 1, upper spectrum).

Elemental analysis of the functionalized material was undertaken to quantify the loading of trisamine and morpholine. Table 1 reports loadings of 0.32 mmol g^{-1} (calculated from back-titration) and 0.40 mmol g^{-1} (calculated from elemental analysis) for morpholine and trisamine respectively. The discrepancy between morpholine loading values determined by elemental analysis and back-titration is attributed to the very low nitrogen content values determined by elemental analysis (error is ca. 0.3%). Conversion of the epoxy groups is low, up to 54% in the case of trisamine. When the reaction was conducted at reflux for 24 h, high conversion of the epoxy group with both morpholine and trisamine was observed, up to 89% for morpholine and 82% for trisamine (see Table 1). Again the difference in values from back-titration and elemental analysis are attributed to the error in the elemental analysis measurement at such low recorded percentages of nitrogen. Conversion of epoxy groups with trisamine and morpholine is comparable to other functional polyHIPE materials [61,62]. Hydrolysis of epoxy groups from GMA-based polyHIPEs during polymerisation has been observed by Barbetta et al. [19]. The hydrolysis of epoxy groups prior to or during polymerisation, which could lead to lower than expected conversions in subsequent reactions, was not investigated here.

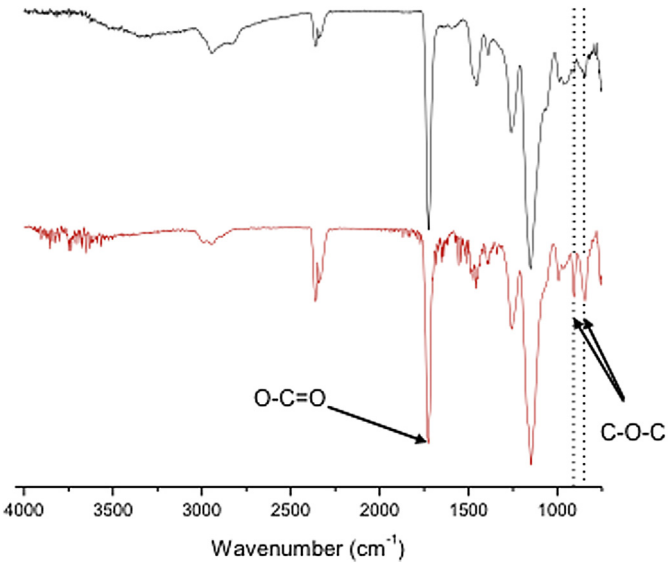


Fig. 1. FTIR spectra: above (black) GMA polyHIPE functionalized with tris(2-aminoethyl) amine, below (red) unfunctionalised GMA polyHIPE. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
GMA-based polyHIPE functionalized with morpholine and trisamine.

Amine nucleophile	% N[a]	Loading (mmol g^{-1})	Conversion (%)[b]
Morpholine[c]	n.d.	0.32[e]	43[e]
Morpholine[d]	n.d.	0.66[e]	89[e]
Trisamine[c]	2.26	0.40[f]	54[g]
Trisamine[d]	3.44	0.61[f]	82[g]

n.d. = not determined.
[a] Results from elemental analysis.
[b] Assuming all epoxy groups from GMA are available for post polymerisation functionalization. Conversion was calculated from the ratio of the loading of amine nucleophile to epoxy content of polyHIPE material.
[c] 2 h reaction at room temperature.
[d] 24 h reaction at reflux.
[e] Calculated from back-titration.
[f] Calculated from elemental analysis.
[g] Assuming no additional crosslinking.

Overall the FTIR, elemental analysis and back-titration data indicate that GMA-based polyHIPEs can be functionalized post-polymerisation with small molecule amines up to a conversion of 89%.

Having established that GMA-based polyHIPEs can be reliably functionalised with nucleophilic amines, we next explored the possibility of attaching a homobifunctional bisamino-PEG linker (*O,O'*-bis(3-aminopropyl)polyethylene glycol) with which to anchor enzymes. GMA-based polyHIPE materials were thus reacted with bisamino-PEG using similar conditions to those employed for the attachment of small molecule amines. The outcome of the reaction was investigated using the qualitative Kaiser test [52], which is used extensively in solid phase peptide synthesis (SPPS) for the detection of primary amines [53]. A positive test (amine groups present) results in the formation of a deep blue coloration on addition of ninhydrin to the polymer [63]. Advantages of this technique are that it is relatively quick, and small quantities of amine groups can be detected [53]. PEGylated GMA polyHIPEs were reacted with ninhydrin and un-functionalized GMA polyHIPEs were used as a control. As can be seen in Fig. 2 the PEGylated polyHIPE material changed to a grey-blue colour (in the web version) on the addition of ninhydrin, indicating the presence of primary amines [53,63], in comparison to the control which remained white in colour.

An amine-reactive fluorescent probe, fluorescein 5(6)-isothiocyanate (FITC), was next reacted with PEGylated polyHIPE to further indicate the presence of amine groups within the material. FITC reacts selectively with amine nucleophiles under alkaline conditions forming thiourea bonds [54]. Monolithic samples of GMA-based polyHIPE were functionalized with bisamino-PEG then reacted with FITC in an alkaline aqueous buffer system. As can be seen in Fig. 3 the typical green (in the web version) fluorescence of FITC is visible when the PEGylated material was irradiated under UV light. Green fluorescence was not observed for the unfunctionalized control; this indicates that FITC is covalently bound to pendent primary amine groups from the bisamino-PEG linker group on the monolith.

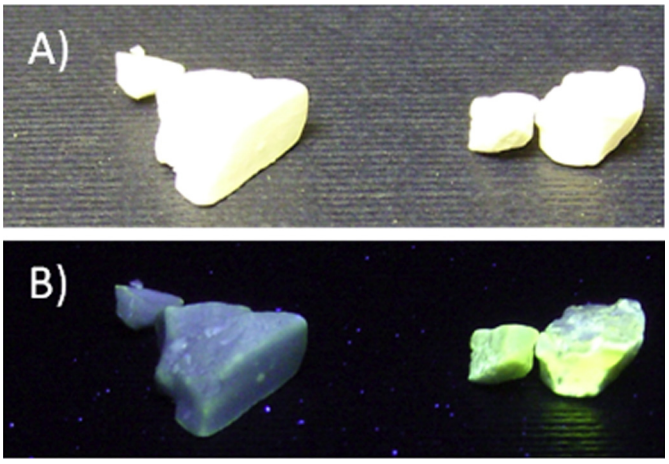


Fig. 3. Reaction of monolithic GMA polyHIPE material with FITC: A) Image in natural light, left: GMA polyHIPE (control); right: PEGylated polyHIPE. B) Image taken with samples illuminated under UV light ($\lambda = 254$ nm) left: GMA polyHIPE (control); right: PEGylated polyHIPE.

Spectroscopic evidence for the covalent attachment of bisamino PEG was provided by High Resolution Magic Angle Spinning (HR-MAS) NMR spectroscopy. This technique involves swelling a crosslinked polymer in a good solvent and spinning at a moderate speed (1–3 kHz) at an angle of ~54° relative to the magnetic field direction, to reduce significantly the line broadening resulting in high resolution NMR spectra [64]. It is particularly useful for obtaining information about the functionalization of crosslinked polymers, as moieties attached to the surface of insoluble supports are more mobile than the support itself [64,65]. Hence, when the insoluble support is swollen, the local environment of these moieties approaches that in an isotropic solution, reducing line broadening [66]. Line widths of less than 4 Hz for compounds immobilized onto insoluble supports have been observed for ^{1}H NMR spectra [67,68]. A crosslinked polymer with pendent PEG chains is particularly well suited to HR-MAS NMR spectroscopy due to the high mobility of the PEG chain ends [64,69,70]. In addition, 2D HR-MAS NMR spectroscopy of solvent swollen gels has also been performed [71], and recently van Camp

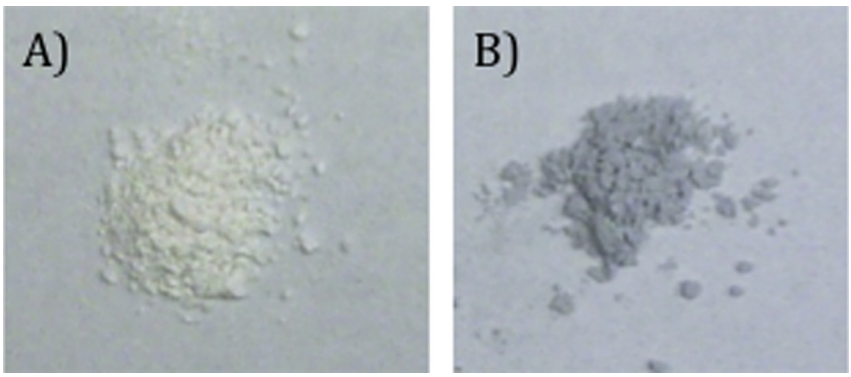


Fig. 2. Powdered polyHIPE materials following reaction with ninhydrin (Kaiser test [52]): A) GMA polyHIPE (control); B) PEGylated polyHIPE.

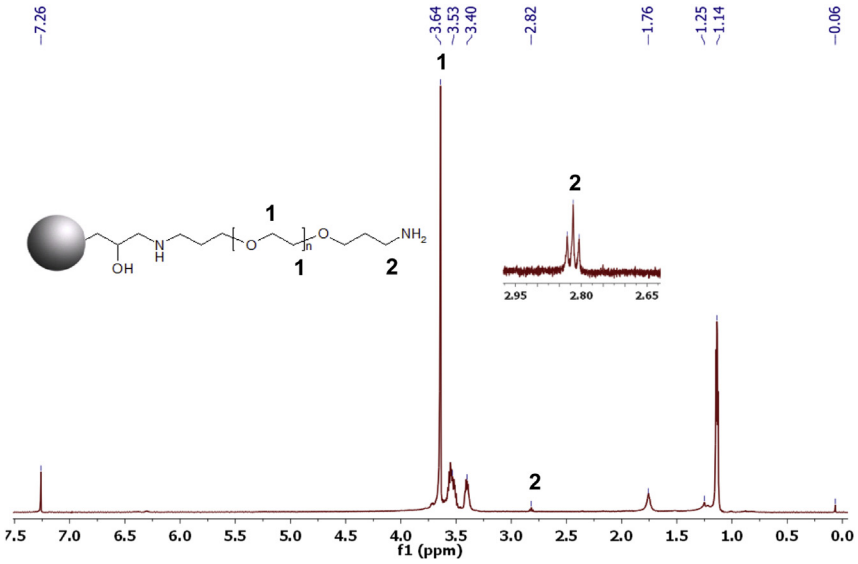


Fig. 4. ^{1}H HR-MAS NMR spectrum of swollen PEGylated GMA polyHIPE at 500 MHz in $CDCl_3$. Inset (left) is molecular structure of PEGylated GMA polyHIPE, numbered to indicate relevant protons that match peaks in the NMR spectrum. Inset (right) is a magnified section of the spectrum showing the peak at 2.82 ppm.

et al. [72] have shown that cryogels functionalized with PEG can be monitored by 1D and 2D HR-MAS NMR spectroscopy.

Using this technique, the functionalization of GMA polyHIPE with bisamino-PEG was monitored. Bisamino-PEG functionalized GMA polyHIPE was swollen in $CDCl_3$ for 30 min prior to acquisition of the ^{1}H HR-MAS NMR spectrum. As can be seen in Fig. 4, the polymer backbone is still clearly visible in the spectrum. Peaks are present from the PEG chain immobilized on the polyHIPE surface; a singlet at 3.64 ppm (peak 1) corresponding to the glycol protons is significantly more intense than the peak corresponding to the polymer backbone, which is indicative of the increase in the local mobility of the PEG chain attached to the polyHIPE surface [66]. In addition to this peak, a triplet at 2.82 ppm corresponding to CH_2–$C\underline{H}_2$–NH_2 on the terminus of the PEG chain is also visible within the spectrum (see Fig. 4 inset, peak 2). This peak is not observed in the spectrum of the polymer backbone indicating that it is not due to solvents or any impurities within the material. These peaks are in good agreement with the solution phase NMR spectrum of this linker group.

2D ^{1}H NMR correlation spectroscopy (COSY) HR-MAS NMR spectroscopy was undertaken on solvent swollen PEGylated polyHIPE materials to access the through-bond proton coupling of the PEG chains. As can be seen in Fig. 5, the peak at 2.82 ppm (peak 2) is coupled to the peak at 1.76 ppm (peak 3). This indicates that the peak at 1.76 ppm corresponds to $C\underline{H}_2$–CH_2–NH_2 on the terminus of the functionalized PEG chain, which is masked in the 1D NMR spectrum by the water solvent peak. As far as can be determined this is the first example of the successful application of 1D and 2D

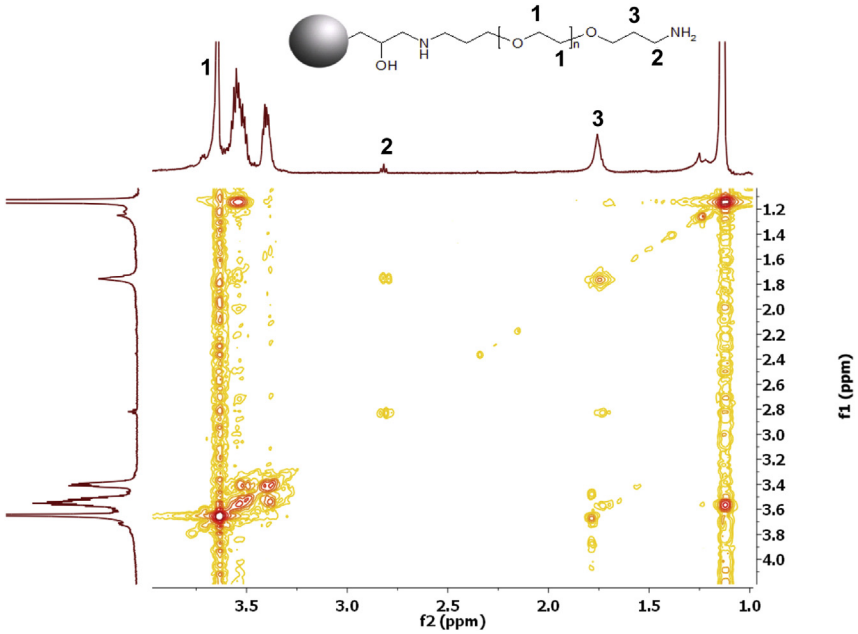


Fig. 5. 2D COSY ^{1}H HR-MAS NMR spectrum of swollen PEGylated GMA polyHIPE at 500 MHz in $CDCl_3$. The molecular structure of PEGylated polyHIPE is shown, numbered to indicate relevant protons that match peaks in the COSY spectrum.

Table 2
XPS data of amine-functionalised GMA polyHIPEs.[a]

Atomic %	GMA[b]	Trisamine[c]	PEG[d]
O 1s	28.8 ± 0.7	24.0 ± 0.1	27.3 ± 0.7
C 1s	69.6 ± 0.8	67.8 ± 0.3	70.7 ± 1.3
N 1s	0.4 ± 0.7	8.0 ± 0.2	1.4 ± 0.1

[a] Three repeats were taken for each sample.
[b] GMA polyHIPE.
[c] Tris(2-aminoethyl) amine functionalized GMA polyHIPE (24 h reaction time).
[d] Bisamino-PEG functionalized polyHIPE.

^{1}H HR-MAS NMR spectroscopy for the assessment of the functionalization of polyHIPE materials.

Anticipating that the surface concentration of amino groups following attachment of bisamino-PEG would be low, quantification of loading was undertaken using the so-called Fmoc number determination method. PEGylated polyHIPE was reacted with 9-fluorenylmethyl chloroformate (Fmoc-Cl, see Scheme 2), the Fmoc group was subsequently removed by reaction with piperidine and the absorbance at 301 nm of the Fmoc-piperidine derivative was measured. The loading of Fmoc was subsequently determined using the Beer–Lambert law. The Fmoc loading was thus determined to be 0.12 mmol g^{-1}. Overall these results show that the conversion of the epoxy groups (original loading 4.8 mmol g^{-1}) is only 2%. This could possibly be due to the high exclusion volume of the PEG chains in aqueous solution on the polyHIPE material preventing other PEG chains from attaching to adjacent epoxy groups on the material's surface [48].

3.2. X-ray photoelectron spectroscopy (XPS) of functionalized GMA/EGDMA PolyHIPE materials

GMA and functionalized GMA polyHIPEs were analysed by XPS to quantify the amount of nitrogen on the materials' surface as well as to determine the chemical environment of atoms at the surface. Table 2 lists the calculated atomic composition of the materials' surface relative to the respective C 1s peak. The atomic composition data of the trisamine functionalized GMA polyHIPE surface in comparison to the unfunctionalized GMA polyHIPE surface (see Table 2) shows a 20× increase in the N 1s peak. This is indicative of the successful covalent surface functionalization of the polyHIPE with trisamine.

Fig. 6 shows the high-resolution XPS spectrum from a GMA polyHIPE surface showing the C 1s peak. There are three distinct chemical environments corresponding to the C 1s peak. C1 component (285.0 eV, full width half maximum (FWHM) 1.3 eV) is assigned to C–C, C–H and C=C bonds; C2 (286.5 eV, FWHM 1.2 eV) is assigned to hydroxyl and ether bonds (C–OH and C–O–C) and C3 (288.7 eV, FWHM 1.4 eV) to (meth)acrylate ester bonds (O–C=O) [73,74]. The larger area of the C2 peak compared to C3 reflects the contribution from the GMA epoxide C–O bonds (this sample has 75 mol% GMA) plus possible polyether moieties from residual surfactant. The chemical shifts of the C 1s peak is representative of polymerised GMA and is similar to the high resolution XPS spectrum of the C 1s peak of poly(methyl methacrylate) [73].

The N 1s peak in the high-resolution XPS spectrum from the trisamine functionalized polyHIPE surface shows that nitrogen is in two chemical environments; binding energies of 398.9 eV (FWHM 1.3 eV) and 401.4 eV (FWHM 1.4 eV) in a ratio of 3:1 were obtained (see Fig. 7). The two chemical environments correspond to non-protonated (NH_2) and hydrogen bonded/protonated ($-NH_2/NH_3^+$) amine species [74,75]. As trisamine is present in excess compared to the epoxy content of the polyHIPE, it is assumed that the majority of trisamine molecules have reacted with only one epoxide group. The high-resolution XPS spectrum of the N 1s peak supports this assumption. The chemical shift of the N 1s peak complements the wide survey XPS scan (not shown) in indicating the successful surface functionalization of GMA polyHIPE with trisamine.

Table 2 lists the calculated atomic composition of PEGylated and unfunctionalized GMA polyHIPE surface relative to the respective C 1s peak in the wide survey scan XPS spectra. Analysis of the atomic composition of the bisamino-PEG functionalized GMA polyHIPE surface in comparison to the unfunctionalized GMA polyHIPE

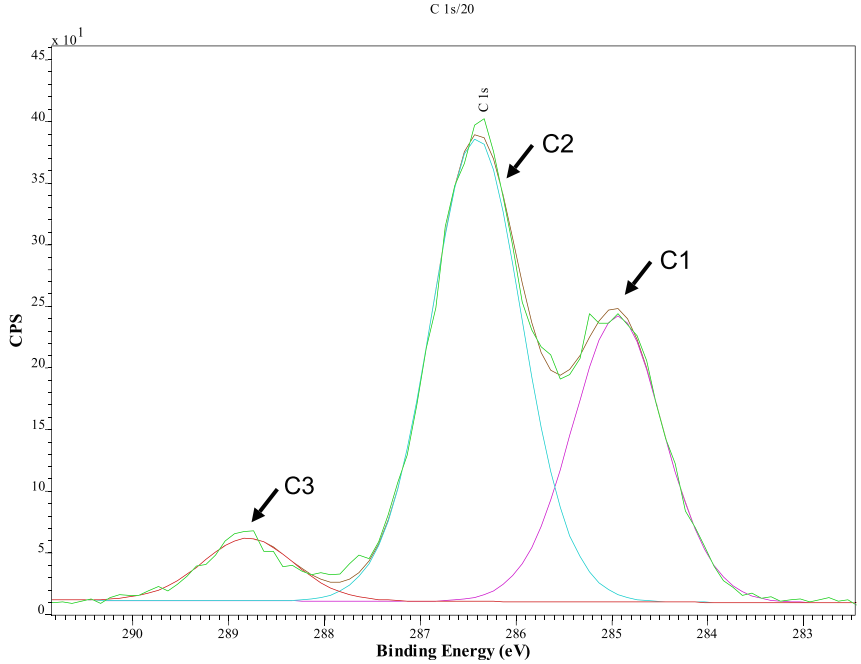


Fig. 6. High-resolution XPS spectrum from a GMA/EGDMA polyHIPE surface showing the C 1s peak. Internal structures corresponding to atoms in different chemical environments have been shown (see text for assignments). CPS is counts per second.

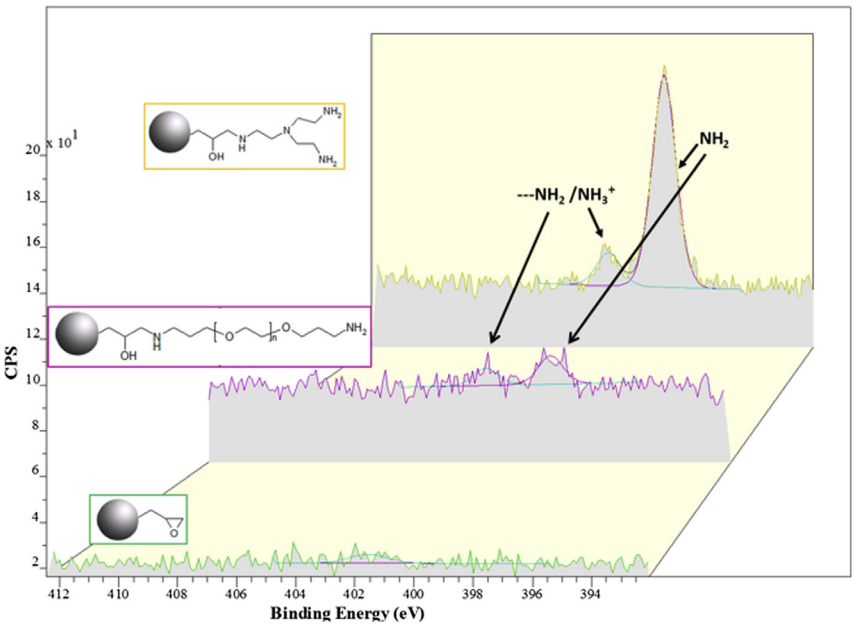


Fig. 7. High resolution XPS spectrum from surface of polyHIPE materials showing N 1s peak above. Top: tris(2-aminoethyl) amine functionalized polyHIPE surface (24 h reaction time); middle: bisamino-PEG functionalized polyHIPE surface; bottom: GMA polyHIPE surface. Internal structures corresponding to atoms in different chemical environments have been shown.

surface shows a 3.5 × increase in the N 1s peak. The low nitrogen content on the PEGylated polyHIPEs surface is suggested to be due to the low loading of bis-amino PEG as well as the much smaller atomic % of nitrogen in bis-amino PEG compared to trisamine. Nonetheless, the small increase in nitrogen content is indicative of the covalent surface functionalization of the polyHIPE with PEG. The high resolution XPS spectrum of the bisamino-PEG functionalized polyHIPE surface indicates that the N 1s peak is in two chemical environments (399.0 eV and 401.7 eV, see Fig. 7) corresponding to non-protonated (NH_2) and hydrogen bonded/protonated ($-NH_2/NH_3^+$) amine species [74,75]. This is similar to the observation with trisamine functionalized polyHIPE, although the intensity of the peak is much lower due to the lower loading of PEG.

3.3. Proteinase K immobilization onto GMA-based PolyHIPE materials

Proteases are of great interest for the production of enantiomerically pure peptides under mild reaction conditions [23], consequently the immobilization of proteinase K onto GMA-based polyHIPEs was investigated. It has been observed that immobilization can stabilise proteases in comparison to the enzyme used in solution [23]. Enzyme immobilization onto polyHIPE materials is a relatively under-studied area of research and only lipases have been studied to any extent previously [40–43].

The assay used for monitoring the activity of Pro K immobilized polyHIPE material was the hydrolysis at pH 9.0 of N-acetyl-L-tyrosine ethyl ester. This assay was chosen as it is a continuous electrochemical assay. Pro K was immobilized directly onto GMA-based photopolymerised polyHIPE, using the reaction between the primary amine groups present in lysine residues on the surface of the enzyme with epoxy groups present within the crosslinked polymer material. In addition to the direct attachment of the enzyme onto the polymer, bisamino-PEG was used for the covalent immobilization of the enzyme. Pro K was immobilized onto the PEGylated polyHIPE material via 'activation' of the material with glutaraldehyde [54–56], then reduction with sodium cyanoborohydride (Scheme 3) [54]. This reduction step is necessary to improve the stability of enzymes immobilized on insoluble carrier materials [76]. Sodium cyanoborohydride has been observed not to result in any reduction in activity when used in conjunction with proteins [54].

The activity of Pro K in solution was determined to be 53,000 U/g of lypophilized enzyme using the pH stat assay. The activity of Pro K immobilized directly onto the polyHIPE was measured as 3.6 U/g of polyHIPE. Attempts to determine the enzyme loading using a Bradford assay were not successful (unreliable data obtained), therefore it is not possible to tell whether there is a low enzyme loading or if the enzyme has been substantially deactivated. The activity of pro K immobilized onto PEGylated GMA-based polyHIPE was measured at between 51 and 78 U/g of polyHIPE, indicating that the PEG linker group had a significantly positive effect on the immobilization of the protease in an active form. Again, as the enzyme loading could not be accurately determined, it is not certain whether the higher activity of the PEGylated material is due to an increase in enzyme loading or due to a reduction in the inactivation of the enzyme on immobilization. The material was reused two more times, washing the material with UHP water between assays, and activities of between 33 U/g–55 U/g and 19 U/g–24 U/g respectively were found. The lowering of the activity between assays suggests either leaching of the protease from the polyHIPE, indicative of some adsorbed enzyme or the inactivation of the enzyme on successive washes.

4. Conclusions

Photopolymerised GMA-containing polyHIPE materials have been post-functionalized with a range of amine nucleophiles. It was observed that near quantitative conversion (up to 89%) of epoxy groups could be achieved by conducting the reaction under reflux for 24 h. Attachment of a hydrophilic homobifunctional PEG linker group was successful, albeit with only ~2% conversion of epoxy groups after reaction for 24 h. This was attributed to the large exclusion volume of PEG chains preventing other PEG chains in

solution attaching covalently to the polyHIPE surface. ^{1}H HR-MAS NMR spectroscopy showed that attachment of bisamino-PEG was successful, as indicated by the increase in intensity at 3.6 ppm as a result of glycol protons of the PEG chains, as well as a triplet peak at 2.6 ppm corresponding to $\underline{CH_2}$–NH_2 of the linker groups. In addition to this, a Kaiser test and reaction with a fluorescent probe, FITC, indicated that primary amine groups were present for further functionalization. Pro K was immobilized directly and via the PEG spacer onto GMA polyHIPE material. The activity of Pro K immobilized directly onto the polymer was low (3.6 U/g and 0 U/g), and it could not be determined whether this was due to inactivation of the enzyme on immobilization or low enzyme loading. In contrast, the activity of the Pro K immobilized onto the PEGylated polyHIPE was much higher (51–78 U/g), although on re-use the activity of the immobilized enzyme decreased significantly.

Acknowledgements

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