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Synthesis and characterization of uniform polyepoxide micrometer sized particles by redox graft polymerization of glycidyl methacylate on oxidized polystyrene and polydivinylbenzene microspheres for enzyme immobilization

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ABSTRACT

Polystyrene template microspheres of narrow size distribution were prepared by dispersion polymerization of styrene in 2-methoxyethanol. Uniform polystyrene/poly(divinyl benzene) composite microspheres were formed by a single-step swelling process of the polystyrene template microspheres with dibutyl phthalate droplets containing divinyl benzene and benzoyl peroxide, followed by polymerization at 73 °C. Uniform poly(divinyl benzene) microspheres of higher surface area were produced by dissolution of the template polystyrene part of the former composite microspheres with methylene chloride. Hydroperoxide conjugated polystyrene and poly(divinyl benzene) microspheres were produced by controlled ozonolysis of these microspheres. Polyepoxide conjugated microspheres were then formed by redox graft polymerization of glycidyl methacylate on the hydroperoxide-conjugated microspheres. Microspheres with different properties, e.g., size, size distribution, shape, surface morphology, surface area, etc., have been prepared by changing various parameters belonging to the ozonolysis and the grafting polymerization processes, e.g., ozonolysis conditions and monomer volume. Trypsin was then covalently bound to the polyepoxide conjugated microspheres by interacting the epoxide groups of the particles with primary amino groups of the enzyme. A comparison between the enzymatic activity of the conjugated and the free trypsin was also established.

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1. Introduction

Polymeric microspheres of narrow size distribution have attracted much attention in many applications such as adsorbents for high-pressure liquid chromatography, calibration standards, spacers for liquid crystals, inks, catalysis, and so forth [1–9]. Polymeric microspheres containing functional groups such as aldehydes, chloromethyls, oxiranes, hydroxyls, and thiols have been used for covalent binding, via various activation methods, of bioactive reagents (e.g., proteins, enzymes, antibodies and oligonucleotides) to the surface of these functional microspheres [1,3,5,10]. The bioactive-conjugated microspheres were then used for various biomedical applications, e.g., specific cell labelling and separation, diagnostics, enzyme immobilization, chromatography, etc. [1,3,5].

Dispersion polymerization is the common method for preparing non-porous microspheres of narrow size distribution in a single step. However, the microspheres formed by this method possess specific properties, e.g., surface morphology and functionality, which can hardly be manipulated [11,12]. Furthermore, uniform microspheres of a diameter larger than approximately 6 μm usually cannot be prepared by dispersion polymerization. These limitations have been overcome by graft polymerization of vinylic monomers on the surface of uniform core microspheres [13–15], and by several swelling methods of PS microspheres with appropriate hydrophobic monomers and initiators, e.g., multi-step swelling, dynamic swelling [16,17], and a single-step swelling [18–23], followed by polymerization of the monomers within the swollen microspheres.

There is much interest among the academic and industrial scientific community in finding new ways to modify the surface of microspheres without changing their bulk properties. The reasons for seeking this kind of modification are many, e.g., changing the surface composition and wettability properties, improving adhesion, protein immobilization, blood compatibility, weathering, protection of microspheres, etc. [24–28]. Numerous methods for surface modification of different microspheres, such as high-energy radiation (e.g. gamma, glow discharge, corona discharge or photo irradiation) [29], ozone exposure [26,30], graft polymerization on core microspheres, etc., have been already published [20,31,32].

The present article describes a simple method for surface modification and functionalization of polystyrene (PS) and

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poly(divinyl benzene) (PDVB) microspheres of narrow size distribution, by conjugating hydroperoxide groups to the microspheres via an ozonolysis process, followed by radical graft polymerization of the monomer glycidyl methacrylate (GMA). The grafted epoxide groups were then used for enzyme, e.g., trypsin, immobilization to increase the stability against an inhibitors and storage, as described previously [32]. A comparison between the activity of the free and the immobilized enzyme was also performed.

2. Experimental

2.1. Chemicals

The following analytical-grade chemicals were purchased from Sigma and were used without further purification: trypsin type IX from bovine pancreas (16300 units/mg protein), α_1-antitrypsin from human plasma, benzoyl peroxide (BP, 98%), dibutyl phthalate (DBP, 99.9%), sodium metabisulfite (97%), sodium dodecyl sulphate (SDS), polyvinylpyrrolidone (PVP, m.w. 360 000), 2-methoxyethanol (HPLC), acetic acid (AA), isopropanol (ISP), ethanol (HPLC), GMA (97%), styrene (99%) and divinyl benzene (DVB, 80%); N-α-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) and phosphate buffered saline (PBS 0.1M, pH 7.4) were purchased from OXOID Limited, England; Bicarbonate buffer (BB 0.1M, pH 8.3) was acquired from Bio-Lab Ltd., Israel. GMA was passed through activated alumina (ICN) to remove inhibitors before use. Water was purified by passing deionized water through an Elgastat Spectrum reverse osmosis system (Elga Ltd., High Wycombe, U.K.). Ozone was produced by passing a current of oxygen through a corona discharge (ozomax. Canada) at voltages from 4.5 to 9 kV.

2.2. Methods

2.2.1. Synthesis of uniform PS microspheres

Uniform PS microspheres of two sizes, 1.3 ± 0.2 μm and 3.23 ± 0.17 μm, were prepared by dispersion polymerization of styrene in 2-methoxyethanol, according to the literature [11,33].

2.2.2. Synthesis of uniform PS/PDVB composite microspheres

Uniform PS/PDVB composite microspheres were formed by a single-step swelling process at room temperature of the PS template microspheres of 1.3 ± 0.2 μm diameter with DBP droplets containing DVB and BP, followed by polymerization of the swollen DVB at elevated temperature. In a typical experiment, uniform composite microspheres of 3.35 ± 0.15 μm diameter were prepared by adding to a 20 mL vial, 10 mL of an SDS aqueous solution (0.75% w/v) and 1.0 mL DBP containing 1.0 mL DVB and 10 mg BP. DBP emulsion droplets in the aqueous continuous phase were then formed by sonication (Sonics and Materials, model VCX-750, Tihorn 20 KHz) of the former mixture for 1 min 3.5 mL of an aqueous suspension of the PS template microspheres of 1.3 ± 0.2 μm diameter (7% w/v) were then added to the stirred former mixture. After the swelling at room temperature of the PS particles was completed (ca. 6 h), and the mixture no longer contained any small droplets of the emulsified DBP, as verified by optical microscopy, the vial containing these swollen microspheres was shaken at 73 °C for 20 h. The formed composite microspheres were then washed by intensive centrifugation cycles with water, ethanol and again water, and then lyophilized.

2.2.3. Synthesis of uniform PDVB microspheres

Uniform PDVB microspheres were prepared by dissolving the PS template polymer of the PS/PDVB composite microspheres with DMF. Briefly, 500 mg of the PS/PDVB composite microspheres of 3.35 ± 0.15 μm diameter, prepared as described in the previous paragraph, were dispersed in 30 mL DMF and then shaken at room temperature for ca. 12 h. The dispersed microspheres were then centrifuged and the supernatant containing the dissolved PS template polymer was discarded. This procedure was repeated four times with DMF. The formed microspheres of 3.12 ± 0.08 μm diameter were then washed further by intensive centrifugation cycles with ethanol and water and then lyophilized.

2.2.4. Synthesis of oxidized uniform PS and PDVB microspheres

In a typical experiment, 1.0 g of the PS or PDVB microspheres of 3.23 ± 0.17 and 3.12 ± 0.08 μm diameter, respectively, dispersed in 50 mL pure water were introduced into a 250 mL round bottom flask. An O_2/O_3 stream containing an ozone output of 4 g h^{-1} was bubbled at room temperature through the dispersed microspheres for 20 min at a flow rate of 1.0 L min^{-1}. The oxidized microspheres were then washed free of excess ozone by intensive centrifugation cycles with water, until the supernatant did not show any indication of free ozone, as measured spectrophotometrically with KI [34]. The effect of ozonolysis time on the microspheres properties and the conjugated hyroperoxide concentration was also studied.

2.2.5. Swelling of the oxidized PS microspheres

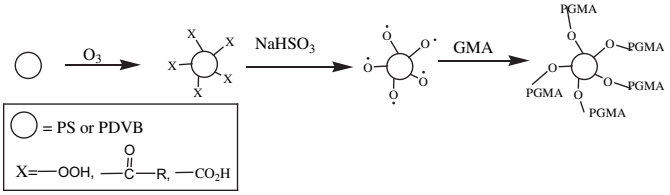
In a typical experiment, 100 mg of the oxidized PS microspheres of 3.23 ± 0.17 μm diameter dispersed in 10 mL of SDS (0.1 g) aqueous solution containing 0.5 mL of GMA were introduced into a 100 mL tube. The mixture was shaken at room temperature for 22 h, and the diameter of the swollen microspheres was then measured by light microscope as described in paragraph 2.3.10. The effect of different volumes of GMA on the swelling of the PS template microspheres was also elucidated.

2.2.6. Redox graft polymerization of GMA on the oxidized PS or PDVB microspheres

Scheme 1 described the synthesis strategy of the PS/PGMA or PDVB/PGMA microspheres. In a typical experiment, 100 mg of the oxidized PS or PDVB microspheres dispersed in 8 mL of SDS (0.1 g) aqueous solution containing 0.5 mL of GMA were introduced into a 250 mL tube. For initiating the graft polymerization process at room temperature, 2 mL of an aqueous solution containing 0.076 mol sodium metabisulfite were immediately added to the former shaken aqueous mixture. The polymerization process was continued for 22 h, followed by washing of the formed PS/PGMA and PDVB/PGMA composite microspheres by intensive centrifugation cycles with water and ethanol and again water, and then lyophilization. The effect of the ozonolysis time duration and the volume of the GMA on the microspheres properties were also elucidated.

2.2.7. Trypsin immobilization on the PS/PGMA composite microspheres

Different amounts of trypsin (0.2, 2 and 5 mg) were added to a 15 mL polypropylene tube containing 10 mg of the PS/PGMA composite microspheres dispersed in 10 mL bicarbonate buffer (BB, pH 8.3). The mixture was then shaken at room temperature for



Scheme 1. A schematic scheme which described the synthesis strategy of the PS/PGMA or PDVB/PGMA microspheres.

22 h. Unreacted epoxide groups were then blocked by adding 2 mL ethanolamine BB solution (0.1%) to the shaken mixture. This mixture was then shaken at room temperature for additional 12 h. The trypsin-immobilized microspheres [PS/PGMA-trypsin] were then washed from unbound trypsin and ethanolamine by intensive centrifugation cycles with PBS (pH 7.4). The activity of the conjugated trypsin was then measured relative to that of the free trypsin (considered as 100% activity).

2.3. Characterization

2.3.1. Conjugated hydroperoxide concentration

The conjugated hydroperoxide concentration was determined according to D.J. Carlsson et al. [35] Briefly, a 20 mL NaI/ISP (14 g/100 mL) solution and 70 mL of an AA/ISP (1: 10 v/v) solution were introduced into a 250 mL round bottom flask containing 100 mg of the oxidized PS or PDVB microspheres. After 30 min reflux, the suspension was cooled to room temperature, and 10 mL of pure water were then added. The I^{3-} concentration formed during the conjugated hydroperoxide decomposition by I^- was determined on a Cary-1E uv-visble spectrometer at 360 nm, based on a calibration curve of I^{3-} concentrations.

2.3.2. Composition of the PS/PGMA and the PDVB/PGMA microspheres

The %PGMA belonging to the PS/PGMA composite microspheres was calculated according to the following equation: % PGMA = ($\%O_{PGMA} \times 100$)/33.8, where 33.8 is the %O of pure PGMA. The $\%O_{PGMA}$ was obtained from the elemental O analysis according to the following equation: $\%O_{PGMA} = \%O_{PS/PGMA} - \%O_{oxidized\ PS}$. The % PS of the PS/PGMA composite microspheres was calculated by reducing from 100 the % PGMA.

The % PGMA belonging to the PDVB/PGMA composite microspheres were calculated similarly, substituting the PS/PGMA microspheres for the PDVB/PGMA microspheres and the PS for the PDVB. The % PDVB of the PDVB/PGMA composite microspheres was calculated by reducing from 100 the % PGMA.

2.3.3. Bound trypsin concentration

The concentration of the immobilized trypsin was measured spectrophotometrically at 650 nm according to the Lowry method [36].

2.3.4. Trypsin activity

The activity of the free and the immobilized trypsin against the substrate BAEE was determined according to Boyer [37]. (One unit (U) of trypsin with BAEE produced a change in A_{253} of 0.001 min^{-1} at pH 7.5, 25 °C in a volume of 3 mL). A known amount of the dried immobilized trypsin microspheres was added to a 13 × 100-mm glass tube containing 2.6 mL PBS. 0.4 mL of the substrate solution (1.9 mg BAEE/mL PBS) was then added, and the mixture was shaken at room temperature for 7 min. The trypsin-conjugated microspheres were then precipitated by centrifugation, and the supernatant was analyzed spectrophotometrically at 253 nm. The activity of free trypsin towards BAEE was determined similarly, substituting the immobilized trypsin for the free trypsin. Inhibition of the free and the immobilized trypsin was accomplished by adding various concentrations of α_1-antitrypsin to the 13 × 100-mm glass tubes containing fixed amounts of free or immobilized trypsin dispersed in 2.6 mL of PBS. The mixture was then shaken at room temperature for 10 min. The activity of the α_1-antitrypsin-inhibited free or conjugated trypsin was then measured with BAEE according to the previously described procedure (8.6 mg α_1-antitrypsin will inhibit 1 mg trypsin with an activity of 10 000 BAEE units mg^{-1} proteins) [37].

2.3.5. Oxygen analysis

Oxygen analysis (by converting organic oxygen into carbon monoxide) of the various microspheres was performed using an elemental analysis instrument; model EA1110, CE Instruments, Thermoquast. The reported O values are an average of measurements performed at least four times for each of the tested microspheres and have a maximum error of about 1%.

2.3.6. SEM

The surface morphology and dry size and size distribution of the microspheres were characterized with a FEI scanning electron microscope (SEM) Model Inspect S. For this purpose, a drop of dilute microspheres' dispersion in water was spread on a glass surface and then dried at room temperature. The dried sample was coated with gold in vacuum before viewing under SEM. The average size and size distribution of the dry microspheres were determined by measuring the diameter of more than 100 microspheres with image analysis software from AnalySIS Auto (Soft Imaging System GmbH, Germany).

2.3.7. BET

The surface area of the various microspheres was measured by the Brunauer-Emmet-Teller (BET) method [38], Gemini III model 2375, Micrometrics. The reported surface area values are an average of measurements performed at least four times for each of the tested microspheres and have a maximum error of about 5%.

2.3.8. TGA

The thermal behaviour of the various microspheres was measured by Thermogravimetric Analysis (TGA). The analysis was performed with a TC15 system equipped with TGA, model TG-50, Mettler Toledo. The analysis was performed with dried samples of about 5 mg in a dynamic nitrogen atmosphere (200 mL min^{-1}) with a heating rate of 10 °C min^{-1}.

2.3.9. FTIR

Fourier Transform Infrared (FTIR) analysis was performed with a Bomem FTIR spectrophotometer; model MB100, Hartman & Braun. The analysis was performed with 13 mm KBr pellets that contained 2 mg of the detected material and 198 mg KBr. The pellets were scanned over 200 scans at a 4 cm^{-1} resolution.

2.3.10. Light microscope

Optical microscope pictures were obtained with an Olympus microscope, model BX51. The particles average size and size distribution were determined by measuring the diameter of more than 100 particles on optical micrographs with image analysis software from AnalySIS Auto (Soft Imaging System GmbH, Germany).

3. Results and discussion

Previous studies with PS, and other polymers (e.g. polypropylene, polyethylene, polyethylene terephthalate and polyurethane) demonstrated the generation of several oxygen-containing groups, e.g., hydroperoxides, ketones and acids, as a consequence of their exposure to ozone [39–41]. Previous studies in our research group described graft polymerization at 70 °C of chloromethylstyrene and acrylonitrile on ozone-oxidized P(styrene-DVB) microspheres [20]. In the present study, ozone induced graft polymerization was used for functionalization of uniform PS and PDVB microspheres with epoxide groups. During this ozonolysis process, the PS and PDVB microspheres were oxidized by ozone which was bubbled through an aqueous dispersion containing these microspheres. This treatment results in the formation of oxygen-containing groups such as hydroperoxides, ketones, hydroxyls and carboxylates conjugated

Table 1
Influence of the ozonolysis time on the concentration of the conjugated hydroperoxide groups.[a]

Microspheres Type	Ozonolysis Time (min)	[Conjugated Hydroperoxides] (mmol/g)
PS	3	0.064
	20	0.076
	120	0.078
PDVB	3	0.058
	20	0.072
	120	0.073

[a] The synthesis and the ozonolysis procedure for different time periods were accomplished according to the experimental part.

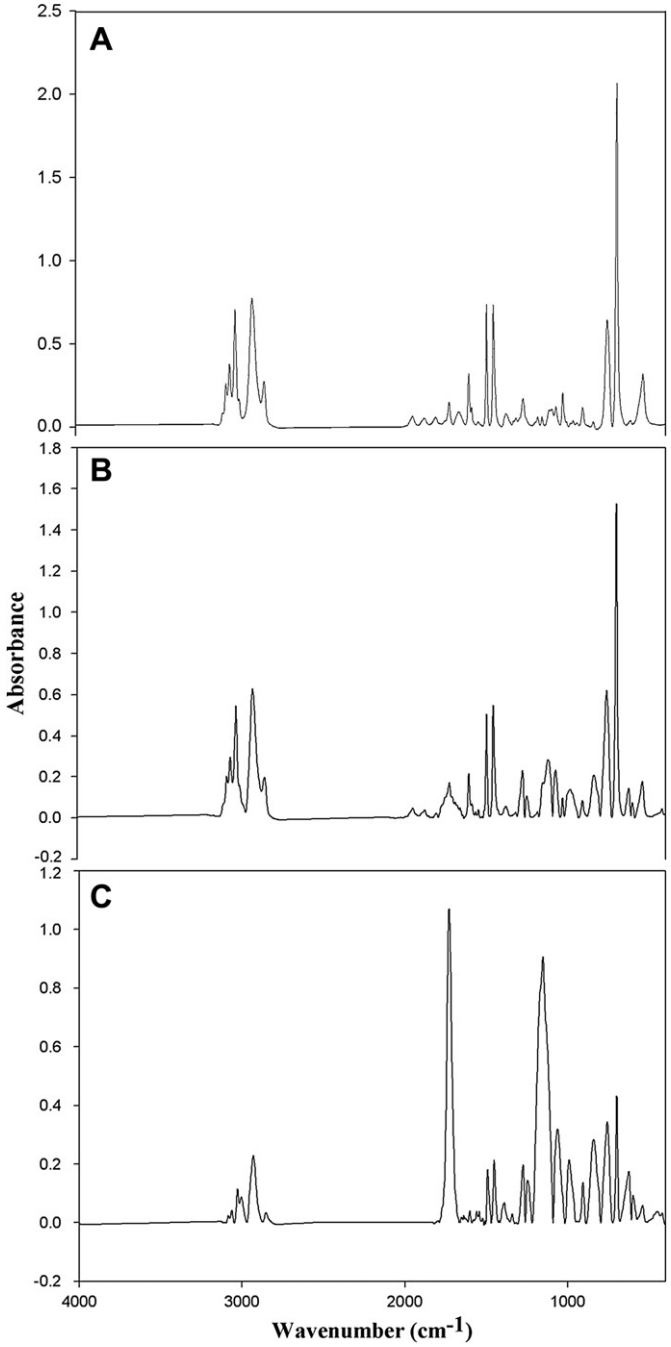


Fig. 1. FTIR spectra of the PS microspheres (A), the oxidized PS microspheres (B) and the PS/PGMA composite microspheres (C).

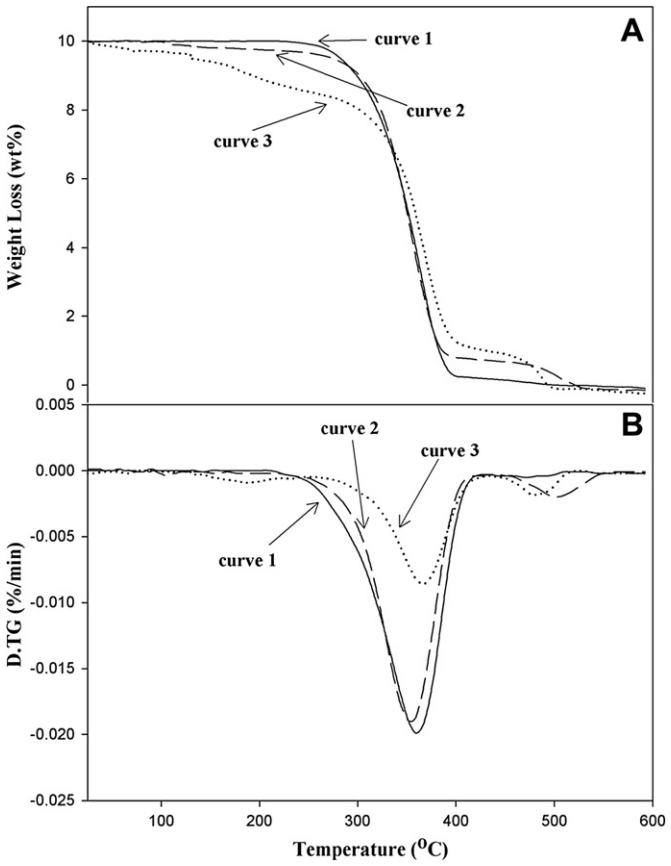


Fig. 2. TGA (A) and DTG (B) thermograms of the PS microspheres (curve 1), the oxidized PS microspheres (curve 2) and the PS/PGMA composite microspheres (curve 3).

microspheres [34]. The conjugated hydroperxides coupled with $NaHSO_3$ have been used in this study as redox initiators for the graft polymerization at room temperature of GMA on these microspheres. The special features of such a graft polymerization are very short induction period, a relatively low energy of activation and the low temperature required. A literature search has revealed that various peroxide/sodium metabisulfite redox pairs are very common as redox initiators in water [42,43]. Bajpai and co-workers proposed that the reductant in the aqueous redox polymerization of acrylamide using potassium permanganate/sodium metabisulfite as redox initiator is the bisulfite anion (HSO_3^-), which is in equilibrium with metabisulfite anion [44]. The addition of an aqueous solution of sodium metabisulfite to the hydroperoxide-conjugated PS or PDVB microspheres dispersed in water results probably in breakage of the conjugated hydroperoxide groups to form conjugated alkoxyl radicals, bisulfite radicals and hydroxyl anions. In the presence of GMA, the conjugated alkoxyl radicals initiate the graft polymerization, while the bisulfite radicals, which are significantly less reactive than the hydroxyl radicals, may initiate homopolymerization in the aqueous continuous phase. The resulting PGMA grafted particles were washed off excess reagents including PGMA nanoparticles of approximately 40 ± 15 nm produced in the aqueous continuous phase by several centrifugation cycles with water and ethanol. The washed PGMA grafted particles were then dried by lyophilization.

3.1. Influence of the ozonolysis time on the properties of the microspheres

Extensive study was done in order to estimate the optimal ozonolysis duration on the PS (3.23 ± 0.17 μm) and PDVB

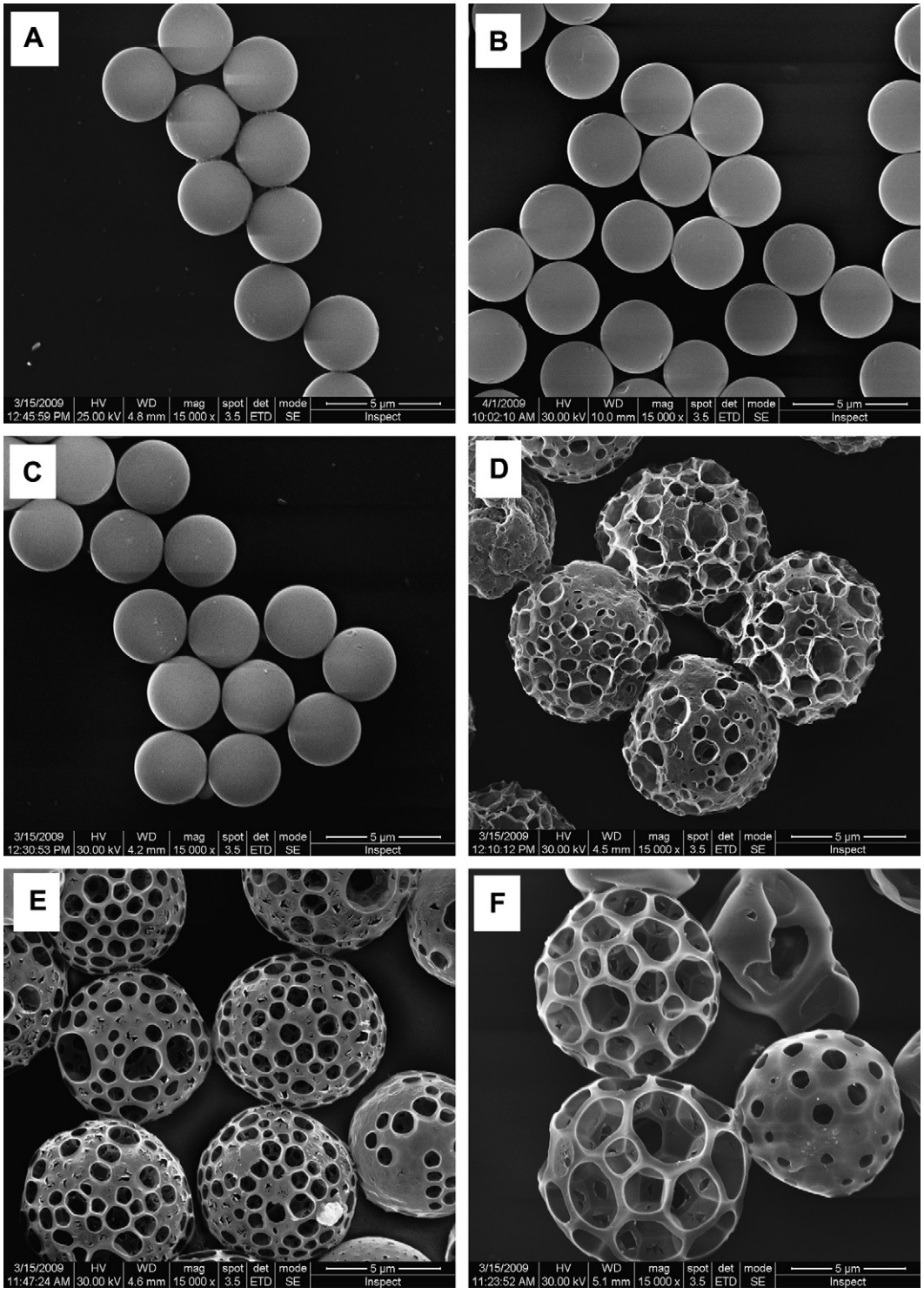


Fig. 3. SEM photomicrographs of the PS microspheres (A), the oxidized PS microspheres (B) and the PS/PGMA composite microspheres prepared by redox graft polymerization of increasing volumes of GMA, 0.1 (C), 0.3 (D), 0.5 (E) and 1.5 (F) mL, on the oxidized PS microspheres of 3.23 ± 0.17 μm diameter, according to the experimental part.

microspheres (3.12 ± 0.08 μm). It was found that the longer the ozonolysis duration, the higher is the concentration of the conjugated hydroperoxide groups. However, on the other hand, under our experimental conditions ozonolysis duration longer than approximately 30 min caused morphology damage and a significant drop in the mechanical properties of the microspheres due to a polymer degradation process; the longer the ozonolysis time the higher is the damage [32]. Table 1 illustrates the influence of the ozonolysis duration on the concentration of the conjugated hydroperoxides. For example, after 3, 20 and 120 min ozonolysis of the PS and the PDVB microspheres, 0.064, 0.076, 0.078 and 0.058, 0.072 and 0.073 mmol/g conjugated hydroperoxide groups, respectively were formed. Based on these results the optimal ozonolysis duration in this study was chosen to be 20 min, yielding 0.076 and 0.072 mmol of conjugated hydroperoxides/g PS or PDVB microspheres, respectively, so that sufficient hydroperoxide groups

Table 2
Influence of the GMA volume on the average diameter and size distribution of the swollen PS microspheres.[a]

V_{GMA} (mL)	Microspheres' Diameter (μm)
Absence of GMA	3.23 ± 0.17
0.1	3.50 ± 0.10
0.3	7.06 ± 0.09
0.5	7.74 ± 1.01
1.5	8.20 ± 0.08

[a] PS template microspheres of 3.23 ± 0.17 μm diameter were swollen with different volumes of GMA according to the experimental part.

Table 3
Influence of the GMA volume on the weight %O and the composition of the PS/PGMA composite microspheres.[a]

V_{GMA} (ml)	%O	PS/PGMA Microspheres' composition	
		%PGMA	%PS
PS only	0	0	100
0.05	3.61	10.67	89.33
0.1	4.06	12.02	87.98
0.3	6.23	18.45	81.55
0.5	8.00	23.67	76.33

[a] The PS/PGMA composite microspheres were prepared by redox graft polymerization of different volumes of GMA on the oxidized PS microspheres of 3.23 ± 0.17 μm diameter, according to the experimental part.

for the graft polymerization were obtained with minimal morphological and mechanical damage to the microspheres [32].

3.2. PS/PGMA composite microspheres

Fig. 1 shows the FTIR spectra of the PS microspheres (A), the oxidized PS microspheres (B) and the PS/PGMA composite microspheres (C). The FTIR spectrum of the PS microspheres demonstrates absorption peaks at 1492 and 3000–3100 cm^{-1} corresponding to the aromatic CH stretching bands, 2849 and 2922 cm^{-1} corresponding to the CH_2 stretching bands and 700 cm^{-1} belonging to the C–C vibrational band. Ozonolysis of these microspheres leads to increasing concentrations of oxygen-containing groups, as illustrated by the carbonyl peak at ca. 1724 cm^{-1}, ether and hydroxyl peaks at 1000–1200 and hydroperoxide and carboxylic peaks at 3200–3500 cm^{-1}. As expected, the FTIR spectrum of the PS/PGMA composite microspheres composed of peaks belonging to both PS (700 cm^{-1}, 1492 cm^{-1}, 3000 cm^{-1}, etc.) and PGMA (845 and 910 cm^{-1} corresponding to the epoxide vibrational bands and 1726 cm^{-1} corresponding to the carbonyl stretching band).

The PS/PGMA microspheres were prepared by redox graft polymerization of 0.5 mL GMA on the oxidized PS microspheres of 3.23 ± 0.17 μm diameter dispersed in an aqueous continuous phase, according to the experimental part.

Fig. 2 illustrates TGA (A) and DTG (B) thermograms of the PS microspheres (curve 1), the oxidized PS microspheres (curve 2) and the PS/PGMA composite microspheres (curve 3). The TGA thermogram of the PS microspheres (Fig. 3A-1) indicates close to 100% weight loss at temperatures range between approximately 300–400 °C. This weigh loss is probably due to the decomposition of the PS polymer, as indicated by the broad DTG peak at ca. 354 °C (Fig. 3C-1). The TGA and DTG thermograms of the oxidized PS (Figs. 3A-2 and B-2, respectively) show two slopes. The initial slope between 300 and 400 °C indicates a weight loss resulting probably from the decomposition of the part of the oxidized PS microspheres that was not interrupted by the ozonolysis process, while the second slope between 400 and 500 °C may relate to the decomposition of the crosslinked PS part generated by the thermal decomposition of the PS-conjugated hydroperoxides. This behaviour is also confirmed by the DTG curve, by the first peak around 350 °C and the second peak around 490 °C. The TGA thermogram of the PS/PGMA microspheres shows three-slope behaviour. A clearer picture is shown by the DTG curve (Fig. 3B-3), demonstrating a first peak at about 200 °C, related probably to the PGMA decomposition, and the followed other two peaks belonging to the decomposition of the non-oxidized and the crosslinked PS parts, respectively, as described previously. The PS/PGMA microspheres were prepared by redox graft polymerization of 0.5 mL GMA on the oxidized PS microspheres of 3.23 ± 0.17 μm diameter dispersed in an aqueous continuous phase, according to the experimental part.

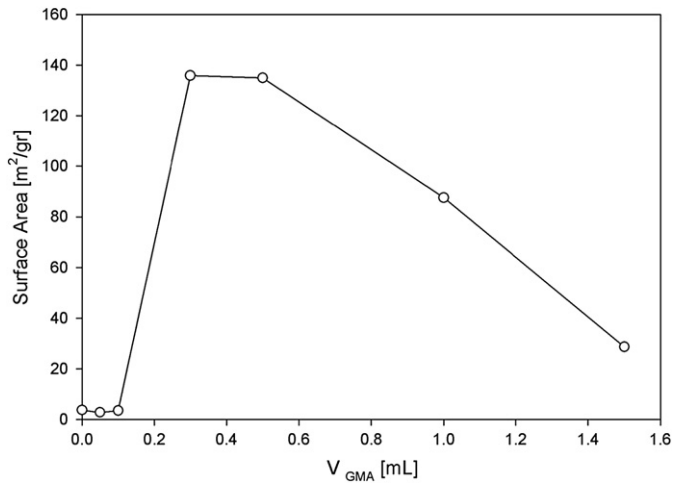


Fig. 4. Influence of the GMA volume on the surface area of the PS/PGMA microspheres.

Fig. 3 shows SEM photomicrographs of the PS microspheres (A) the oxidized PS microspheres (B) and the PS/PGMA composite microspheres produced by redox graft polymerization of increasing volumes of GMA, 0.1 (C), 0.3 (D), 0.5 (E) and 1.5 (F) mL, on the oxidized PS microspheres of 3.23 ± 0.17 μm diameter dispersed in aqueous continuous phase, as described in the experimental part. Fig. 3 shows that the SEM photomicrograph of the oxidized PS microspheres is similar to that of the PS microspheres, i.e., after 20 min ozonolysis the PS microspheres retain their spherical shape, smooth surface morphology and their size and size distribution. This Figure also illustrates that increasing the GMA volume results in increasing size while retaining, more or less, the size distribution of the produced composite microspheres. For example, in the presence of 0.1, 0.3, 0.5 and 1.5 mL GMA, the diameter of the microspheres increased from 3.23 ± 0.17 to 3.33 ± 0.07, 6.70 ± 0.53, 7.07 ± 1.09 and 6.94 ± 1.35 μm, respectively. The increased diameter is probably due to the graft polymerization of the GMA on both the outer surface of the oxidized PS particles as well as within the oxidized PS particles, due the swelling of these template particles with the GMA, as illustrated in Table 2 [19]. Fig. 3 also shows that in the presence of a relatively low volume of GMA, e.g., 0.1 mL, smooth surface morphology was observed. On the other hand, in the presence of increasing GMA volume, e.g., 0.5 mL, pierced shape composed of holes of 0.82 ± 0.21 μm on the PS microspheres were produced. Furthermore, the diameter of the holes of the composite particles increases as the GMA volume rises. This pierced shape is probably due to the washing off step of the non-polymerized GMA molecules. This excess of GMA molecules actually act as a porogen, and in the presence of relatively higher volumes of the non-polymerized GMA larger pores are formed, probably by partial

dissolution of the PS matrix. It should be noted that the PS/PGMA composite microspheres produced in the presence of 1.5 mL GMA or above posses very poor mechanical properties, and on a slight pressure may break into pieces. The surface area of the PS/PGMA microspheres produced in the presence of different volumes of GMA (see Fig. 4) illustrates behaviour similar to the morphology changes shown in Fig. 3. For example, the surface area of the PS/PGMA composite microspheres prepared in the presence of 0.5 mL GMA (135.5 m^2/g) is significantly higher than that prepared in the presence of 0.05, 0.1 or 0.3 mL (3.4, 3.5 and 13.5 m^2/g, respectively). On the other hand, increasing the volume of the GMA above 0.5 mL, e.g., 1.0 and 1.5 mL, resulted in decreasing the surface area from 135.5 m^2/g to 85.5 and 28.7 m^2/g, respectively. This decrease is probably due to the significant increase in the diameter of the holes of the produced in the presence of the higher GMA volumes.

The PS/PGMA microspheres were produced by redox graft polymerization of different volumes of GMA on the oxidized PS microspheres of 3.23 ± 0.17 μm diameter, according to the experimental part.

Table 3 illustrates the influence of the GMA volume on the composition of the PS/PGMA composite microspheres. As expected, this table indicates increase in the %O and the %PGMA of the composite microspheres as the concentration of GMA increases. For example, in the presence of 0.05, 0.1, 0.3 and 0.5 mL GMA, the %PGMA of the PS/PGMA composite microspheres increased from 10.66 to 12.02, 18.45 and 23.67%, respectively.

The %PGMA and PS of the composite PS/PGMA microspheres was calculated from oxygen analysis measurements according to the description in the experimental part.

3.3. PDVB/PGMA composite microspheres

PS/PGMA composite microspheres are soluble in organic solvents such as toluene, methylene chloride, etc. In order to prepare organic stable composite particles the PS microspheres were replaced with the crosslinked PDVB microspheres, according to the description in the experimental part. Fig. 5 displays SEM images of the PDVB microspheres (A), the oxidized PDVB microspheres (B), and the PDVB/PGMA composite microspheres produced by redox graft polymerization of increasing volumes of GMA: 0.3 (C) and 0.5 (D) mL, according to the experimental part.

Fig. 5-A illustrates the porous structure of the PDVB microspheres as compared to the non-porous structure of the PS microspheres (Fig. 3-A). Fig. 5-A & B show that the shape, surface morphology and the size and size distribution of the PDVB microspheres (3.12 ± 0.08 μm) were not significantly influenced by the 20 min ozonolysis process. Fig. 5-C demonstrates that the size and size distribution of the oxidized PDVB were preserved, more or less, by the graft polymerization of 0.3 mL GMA (3.13 ± 0.08 μm) and slightly increased by the graft polymerization of 0.5 mL GMA (3.2 ± 0.09 μm). This small change in the size may be explained by

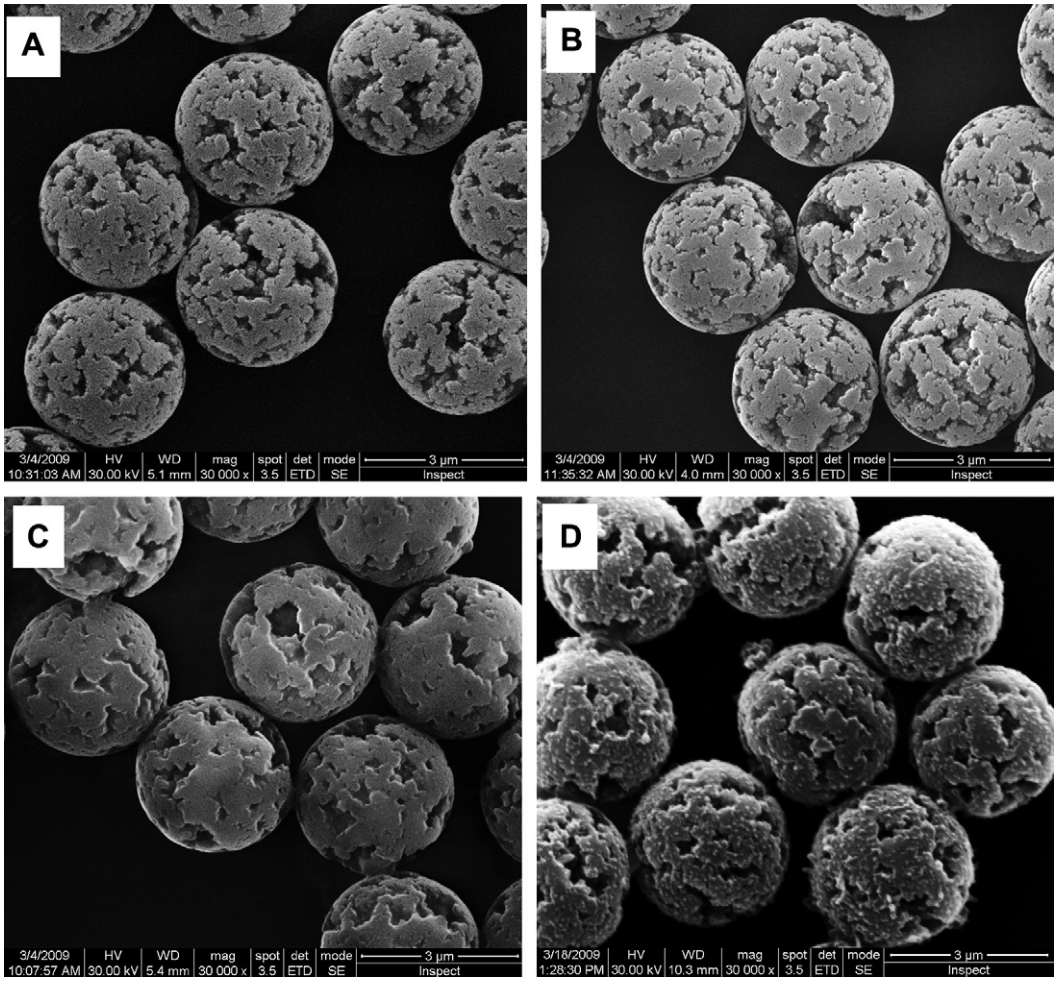


Fig. 5. SEM photomicrographs of the PDVB microspheres (A), the oxidized PDVB microspheres (B) and the PDVB/PGMA composite microspheres prepared by redox graft polymerization of increasing volumes of GMA, 0.3 (C) and 0.5 (D) mL, on the oxidized PDVB microspheres of 3.12 ± 0.08 μm diameter, according to the experimental part.

polymerization of the 0.3 mL GMA within the pores of the porous oxidized PDVB particles, so that the diameter of these particles is preserved. On the other hand, in the presence of 0.5 mL GMA part of the graft polymerization occurred also on the surface of these particles, as can easily be visualized by the increasde roughness of the observed composite particles (Fig. 5-D), resulting by the slight size increase. Support for this behaviour may be obtained by following the change in the surface area of the PDVB/PGMA composite particles formed in the presence of different volumes of GMA, as shown in Fig. 6. Indeed, the surface area of the oxidized PDVB particles decreases from 438.3 to 104.6 m^2/g after graft polymerization of 0.3 mL GMA, probably due to the polymerization within the pores of the PDVB. On the other hand, the surface area of the formed PDVB/PGMA increases from 104.6 m^2/g to 272.0 and 430.1 m^2/g after polymerization with 0.5 or 1.5 mL GMA, respectively, probably due to the increased surface roughness as a consequence of the polymerization of GMA also on the surface of the particles.

The PDVB/PGMA microspheres were produced by redox graft polymerization of different volumes of GMA on the oxidized PDVB microspheres of 3.12 ± 0.08 μm diameter, according to the experimental part.

Table 4 illustrates the influence of the GMA volume on the composition of the PDVB/PGMA composite microspheres. As expected, this table shows increase in the %O and the %PGMA of the composite microspheres as the concentration of GMA increases. For example, in the presence of 0.05, 0.1, 0.3 and 0.5 mL GMA, the %PGMA of the PDVB/PGMA composite microspheres increased from 0.72 to 3.93, 14.35, and 52.63%, respectively.

The %PGMA and PDVB of the composite PDVB/PGMA microspheres were calculated from oxygen analysis measurements according to the description in the experimental part.

3.4. Trypsin immobilization and activity

Trypsin was covalently bound to the polyepoxide PS/PGMA microspheres, according to the experimental part. In order to prevent side reactions between residual epoxide groups and primary amine groups of the inhibitor α_1-antitrypsin, blocking the residual epoxide groups after trypsin immobilization was performed with ethanolamine, as described in the experimental part. Table 5 compares the binding efficiency, the bound trypsin concentrations and the relative activity of the different amounts of trypsin bound to the PS/PGMA microspheres. Table 5 indicates that the trypsin binding efficiency in the the presence of different initial amounts of trypsin is relatively very high, between 87 and 98%. This table also shows that the concentration of the bound trypsin increases as the initial trypsin amount is increased. For example, in the presence of 0.2, 2.0, and 5.0 mg trypsin 19, 176 and 435 μg trypsin was bound to 1 mg of the PS/PGMA microspheres. Table 5 also illustrates that the relative activity towards BAEE of the bound trypsin decreased from 50.4 to 45.4 and 40.5%, respectively as the bound trypsin concentration increased from 19 to 176 and 435 μg /mg microspheres. Usually, the covalent binding of enzymes to polymeric supports leads to a decrease in their activity, but, on the other hand, to an increase in their stability [32]. Indeed, free trypsin lost almost 100% of its activity towards BAEE upon storage at 4 °C of a free trypsin solution (1 mg mL^{-1}) in PBS (0.1 M, pH 7.4). On the other hand, under similar conditions the trypsin bound to the PS/PGMA microspheres lost only 14% of its activity. Fig. 7 also demonstrates the stability of the bound trypsin compared to free trypsin in the presence of different concentrations of the α_1-antitrypsin inhibitor. Curve A in Fig. 7 illustrates the continuous activity loss as long as the [antitrypsin]/[trypsin] mol ratio increased from 1 to 7. At a ratio of 7, the activity loss was approximately 70%. On the other hand, curve B in Fig. 7 demonstrates that under similar conditions the trypsin bound to the PS/PGMA microspheres retained almost all of its activity even at a [antitrypsin]/[trypsin] mol ratio higher than 10.

The binding of the trypsin to the PS/PGMA microspheres, the inhibition with α_1-antitrypsin and the activity measurements against BAEE were accomplished according to the experimental part.

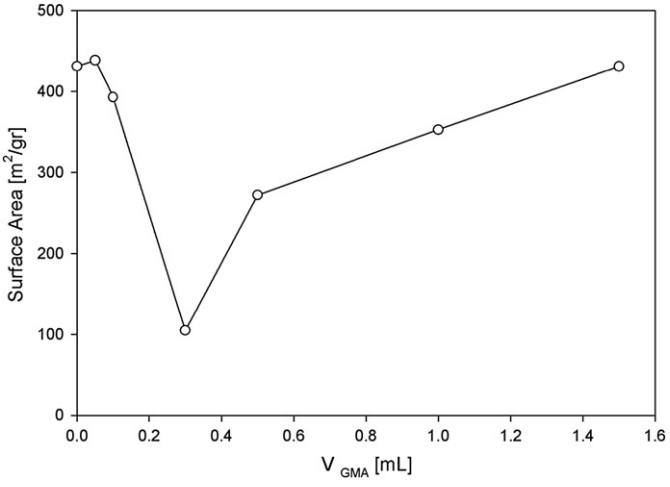


Fig. 6. Influence of the GMA volume on the surface area of the PDVB/PGMA microspheres.

Table 4
Influence of the GMA volume on the weight %O and the composition of the PDVB/PGMA composite microspheres.[a]

V_{GMA} (ml)	%O	PDVB/PGMA Microspheres' Composition	
		%PGMA	%PDVB
PDVB only	0	0	100
0.05	0.24	0.72	99.28
0.1	1.33	3.93	96.07
0.3	4.85	14.35	85.65
0.5	17.79	52.63	47.37

[a] The PDVB/PGMA composite microspheres were prepared by redox graft polymerization of different volumes of GMA on the oxidized PDVB microspheres of 3.23 ± 0.17 μm diameter, according to the experimental part.

4. Summary and future work

This study demonstrates a simple and convenient method for the functionalization of PS and PDVB microspheres by ozonolysis.

Table 5
Trypsin binding efficiency, bound trypsin concentration and bound trypsin relative activity of the PS/PGMA-trypsin microspheres.[a]

Trypsin (mg)	Trypsin Binding Efficiency (%)	[Bound Trypsin] (μg trypsin/mg microspheres)	Bound Trypsin Relative Activity (%)
Free Trypsin	–	–	100
0.2	98.1	19	50.4
2.0	88.0	176	45.4
5.0	87.0	435	40.5

[a] The synthesis and the ozonolysis of the PS/PGMA were performed according to the experimental part. 10 mg of the PS/PGMA composite microspheres dispersed in 10 mL BB were interacted with different amounts of trypsin (0.2, 2.0, and 5.0 mg) according to the experimental part. The bound trypsin activity was measured relative to the same concentration of free trypsin, according to the experimental part.

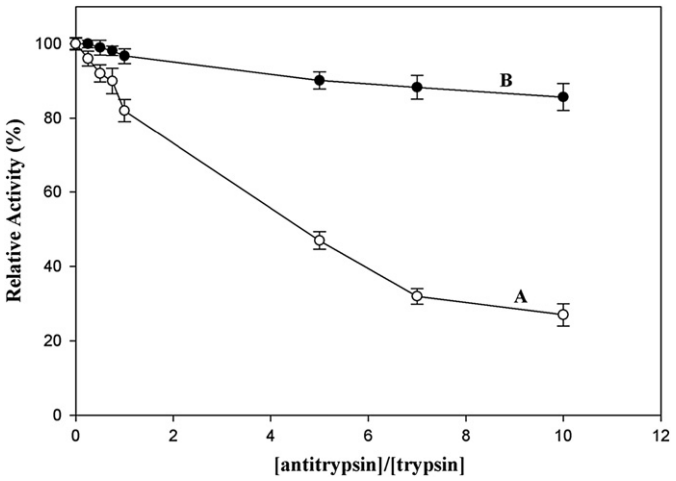


Fig. 7. Relative activity of the free trypsin (A) and the trypsin-conjugated PS/PGMA microspheres (B) as a function of the molar ratio [antitrypsin]/[trypsin].

The ozone treatment of these microspheres promotes the formation of various oxygen-containing groups, e.g., carbonyls, carboxyls, hydroxyls and hydroperoxides. The redox graft polymerization of GMA on the PS and the PDVB microspheres was performed at room temperature by the conjugated hydroperoxides/$NaHSO_3$ redox pair initiator. Stability towards organic solvents was accomplished by replacing the non-porous PS with the porous PDVB microspheres. Initially we assumed that the redox graft polymerization of GMA will occur on the outer surface of the PS microspheres only. However, due to the swelling of the PS particles by the GMA. The polymerization of GMA occurs on both: within the particles as well as on the outer surface of these microspheres. The redox graft polymerization of GMA on the porous PDVB microspheres occurs, as expected, within the pores and on the surface of these particles. The properties of the formed polyepoxide microspheres, e.g., the smooth or pierced shape, size and size distribution, porosity, surface area, surface morphology, etc., were controlled by changing various parameters belonging to the polymerization process, e.g., particles type and GMA concentrations. Trypsin was then covalently bound to the polyepoxide PS/PGMA microspheres. The concentration of the bound trypsin increased as the initial trypsin concentration increases. The covalent immobilization of the trypsin to the polyepoxide microspheres leads to decreased activity against BAEE and increased stability against the inhibitor α_1-antitrypsin and upon storage at 4 °C.

In future work we wish to extend these studies to particles of lower diameters, e.g. below 100 nm, and to the graft polymerization of other monomers such as acrolein and acrylic acid. The optimal systems will then be used for various applications, e.g., coupling of different bioactive amino ligands (proteins, antibodies, enzymes, etc.) to the functional microspheres, via different activation methods, for various biomedical applications [3,5].

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