Mineralization behaviour of some new phema-based copolymers with potential uses in tissue engineering

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This paper reports the mineralization ability of 2-hydroxyethyl methacrylate (HEMA) and 2-methacryloylamido glutamic acid (MAGA) based copolymers incubated in synthetic fluids. MAGA monomer was obtained by organic synthesis and next p(HEMA-co-MAGA) copolymers with different compositions were prepared by bulk radical polymerization using benzoyle peroxide as initiator and ethyleneglycol dimethacrylate as cross-linking agent. The monomer and polymers were further characterized by FTIR-ATR spectroscopy to confirm their structure. Finally, polymers ability to initiate the formation and growth of HA crystals onto their surface in synthetic fluids was proven. SEM analysis showed the formation of apatite-like crystals (calcospherites), fact confirmed also by EDX analysis.

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1. Introduction

The classic biomaterials used in orthopaedic surgery as bone substitutes are mostly inactive biologic biomaterials (osteoconductive materials) [1-7]. These materials serve only as passive support for bone reconditioning [2-8]. These materials could became biologically active, by proteins incorporation which interfere in the osseous morphogenesis (osteoinductive materials). An ideal orthopaedic biomaterial, used for osseous reconstruction by stimulation of osseous tissue growth at the level where the injury appeared, should have biocompatibility and adequate mechanical performances and also should be capable of forming a direct link with the bone (without creating an intermediary fibrous tissue) and stimulating bone reconstruction [4-9]. Currently used metallic materials are biocompatible but very rigid for the organism. Ceramics (calcium phosphates) are brittle and have a high elasticity modulus compared to natural bone. This implies some negative aspects concerning bone fractures. Polymers are used in osseous applications due to their wide chemical composition, to their different mechanical behaviour and because the organic bone matrix are mainly formed by macromolecules Synthetic biocompatible polymers may be an alternative for bone reconstruction as they can serve as matrix for *in vivo* formation of HA. In order to evaluate the capacity of the polymers to promote HA deposition, some *in vitro* methods could be used [11-14]. In 1991, Kokubo [11, 12] developed a simple biomimetic test to reproduce the formation of an apatite layer ex vivo and thereby evaluate the bioactivity of a given material. This test has been widely used since then for the study of biomineralization on different types ofmaterials [5, 7–14] and their ability to form apatite on their surfaces has been correlated with their in vivo bioactivities. This means that the in vivo bone-bonding ability of a given material can be predicted from the apatite formation on its surface when subjected to this test. An acellular protein-free simulated body fluid (SBF) with ion concentrations, pH and temperature nearly equal to those of the human blood plasma is employed as the medium for apatite nucleation. Another possibility is to alternatively immerse the polymers in some calcium and phosphate solutions at 37 °C by the so-called alternate soaking process [13].

PHEMA is one of the most studied polymer for biomedical applications. Various studies have shown that poly (2-hydroxyethyl methacrylate) a polymer widely used as a biomaterial, promotes spherical HA deposits in vitro, when modified by carboxymethylation. HA deposition is due to the presence of numerous anionic sites representing promoters of nucleation. HEMA-based copolymers with various carboxylic and/or other negatively charged groups are intensively studied for their calcification potential and many reports could be found in literature [1-4].

Taking into account the above considerations, this research study was conducted to prepare new polymeric structures based PHEMA capable of in vitro mineralization. Therefore, we have prepared copolymers of 2-hydroxyethyl methacrylate (HEMA) and 2-methacryloylamido glutamic acid (MAGA) with different compositions and evaluated their mineralization ability by incubation in various synthetic fluids.

2. Experimental

2.1. Materials

Glutamic acid hydrochloride and methacryloyl chloride were supplied by Fluka. 2-methacryloylamidoglutamic acid (MAGA) was obtained by organic synthesis [10]. 2-hydroxyethyl methacrylate monomer (HEMA) was obtained from Fluka and distilled under reduce pressure (50 °C and $p=2.1\times10^{-2}$ mmHg) in

the presence of hydroquinone inhibitor and then stored at 4 °C prior to use. Ethylene glycol dimethacrylate (EGDMA) was provided also by Fluka and used without any further purification as cross-linking agent. The initiator used was benzoyle peroxide (BPO) and it was purified by recrystallization from methanol at 40 °C. All other chemicals used were of reagent grade and were purchased from Aldrich.

2.2. Methods

2.2.1. Preparation of MAGA

Briefly, glutamic acid and hydroquinone were dissolved in dichloromethane solution. This solution was cooled down to 0 °C. Next, triethylamine was added to the solution and methacryloyl chloride was poured into this solution under inert atmosphere. This solution was mechanically stirred at room temperature for 1 hour. After this operation, the solution is acidified with concentrated hydrochloric acid (37 %) up to pH = 2. Then, an extraction with acetyl acetate is performed 3 times and finally washing with ethylic ether is achieved. A white insoluble product is formed that finally is filtered and dried. This product is 2-methacryloylamido glutamic acid [10].

2.2.2 Preparation of p(HEMA-co-MAGA) copolymers

A series of p(HEMA-co-MAGA) copolymers were obtained by increasing the concentration of MAGA monomer (5%, 10% and 20% by weight). The copolymers were synthesized by bulk radical polymerization with BPO as initiator (10^{-2} molar) and cross-linked with EGDMA (3 % by weight). The mixture was homogenized by vortexing 15 minutes at 30 Hz and poured in polyethylene moulds (10 mm in diameter and 3 mm in height). Polymerization reactions were carried out in inert atmosphere, at 80 °C, for 10 hours, followed by post-polymerisation at 110 °C, for 4 hours. There were obtained polymeric pellets with flat shape and maximum surface, which were extracted in Soxhlet for 12 h with distilled water to remove any traces of the residual monomer that could negatively influence the in vitro assays.

2.2.3 Characterisation techniques

FTIR spectra were taken on a Schimadzu spectrophotometer 8900 (40 scans and 4 cm^{-1} resolution).

2.2.4 Mineralization assay

For mineralization assay, three samples of each copolymer with different composition were incubated in synthetic body fluid (SBF1x, 12, 15) at pH =7.4, adjusted with tris(hydroxy-methyl) aminomethane (Tris) and hydrochloric acid (HCl), for 14 days, under sterile conditions, in containers with 45 mL of the incubation medium at 37 °C. The incubation medium was changed every 48 h. After incubation, the hydrogels were rinsed with distilled water to remove any traces of salts from the surface and dried at 40 °C for 24 h. The composition of SBF 1x is presented below: Na^+: 142.19 mM, Ca^{2+}: 2.49 mM, Mg^{2+}: 1.5 mM, HCO_3^-: 4.2 mM, Cl^-: 141.54 mM, HPO_4^{2-}, 0.9 mM, SO_4^{2-}: 0.5 mM, K^+: 4.85 mM.

The second incubation method was adapted from literature [13]. Briefly, p(HEMA-co-MAGA) copolymers were immersed in $CaCl_2$ (200Mm/TRIS-HCl) (pH 7.4) aqueous solution at 37 °C for 12 hours, followed by rinsing at 37 °C with distilled water.

After rinsing, the swollen pellets were allowed to swell in Na_2HPO_4 (120 mM) aqueous solution at 37 °C for 12 hours. Through these 2 steps, the formation of hydroxyapatite onto the surface of the polymeric pellets was achieved by alternatively soaking the materials into these solutions.

The presence of mineral crystals onto the surface of the copolymers was evaluated by SEM analysis. The Ca/P molar ratio was investigated by EDX spectroscopy. It was used a JEOL JSM-6301F (JEOL Paris, France), equipped with an EDX microanalysis system, the model Link ISIS (Oxford, Anglia). The samples were dried at 37 °C to constant mass before analysis, and then they were mounted on Pb plots and covered with a thin layer of carbon using a MED 020 Baltec (Balzers, Lichtenstein).

3. Results and discussion

3.1. Preparation of MAGA

The monomer 2-methacryloylamidoglutamic acid was obtained by organic synthesis according to the scheme presented below (Fig. 1).

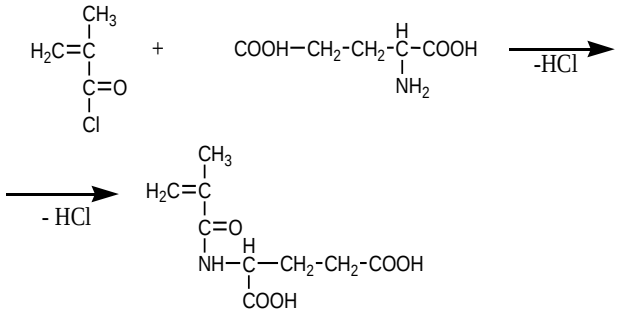

Fig. 1. Chemical reactions to obtain 2-methacryloylglutamic acid.

3.2. Preparation of p(HEMA-co-MAGA) copolymers

There were obtained copolymers HEMA-MAGA with increasing concentration of MAGA monomer (5%, 10% and 20% by weight). The copolymers were obtained as pellets having flat and maximum surface, conditions required for the future *in vitro* assays.

3.3. Characterisation techniques

FTIR spectra showed specific bands for 2-methacryloylamidoglutamic acid monomer (Fig. 2): 1510 cm^{-1} (NHCOR), 3062.7 cm^{-1} (COOH), 538.1 cm^{-1}, 1128.3 cm^{-1} (CH_2CR_2). The specific bands for the copolymer p(HEMA-co-MAGA) were observed and the comparison of the pHEMA and p(HEMA-co-MAGA) spectra was done to better confirm the copolymer structure (spectra not shown).

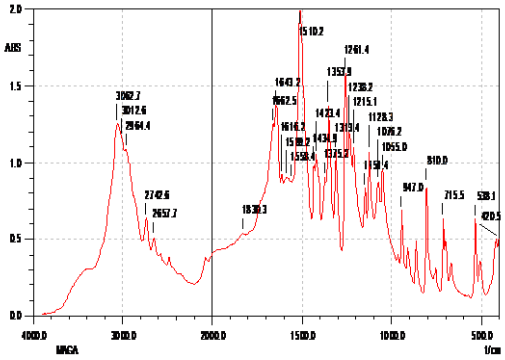

Fig. 2. FTIR spectrum for MAGA monomer.

3.4. Mineralization assay

The crude polymers HEMA-MAGA were analysed by SEM before incubation in body fluids to see further on the clear difference between the incubated and crude materials. The crude samples have asperities and micro-cracks on the surface due to the drying process (Fig. 3).

SEM microphotographs showed the presence of mineral deposits on all the surfaces of incubated materials (both by biomimetic method and alternating soaking process). As it can be seen from these microphotographs (Figs. 4-5), apatite-like crystals are present onto all the surfaces. Their morphology appears as needle-like structures emerging from the copolymer surface. If we try to compare the two incubation methods we may say that the copolymers gave better results when incubated by biomimetic method of Kokubo than the alternating soaking process.

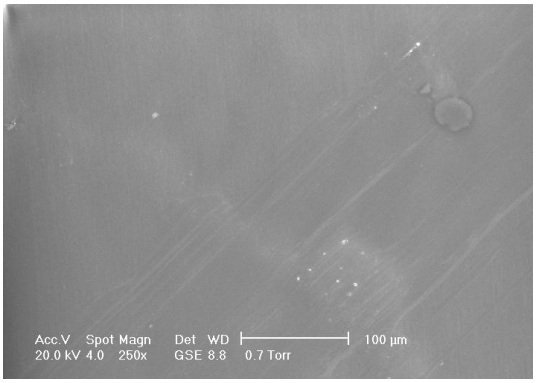

Fig. 3. SEM microphotograph for p(HEMA-MAGA) with 10 wt. % MAGA in feed before in vitro assay.

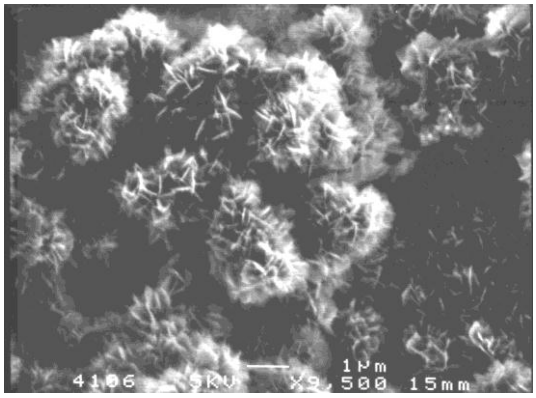

Fig. 4. SEM microphotograph for p(HEMA-MAGA) with 10 wt. % MAGA in feed incubated in SBF 1x.

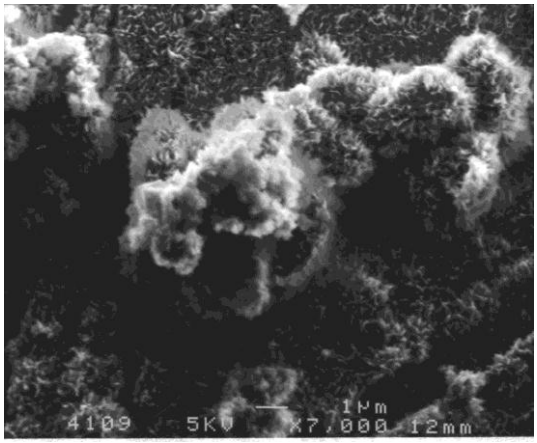

Fig. 5. SEM microphotograph for p(HEMA-MAGA) with 10 wt. % MAGA in feed incubated in alternate solutions.

These results were also sustained by EDX spectra that give the molar ratio Ca/P. EDX spectra showed the presence of calcium, magnesium, oxygen, phosphorus and the ratio Ca/P was very close to the value from HA (1.67). In Fig. 6 there are shown the Ca/P ratios for p(HEMA-co-MAGA) with 10 % MAGA in feed.

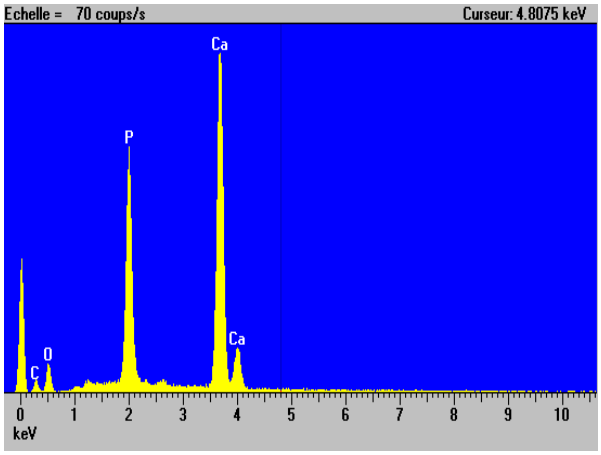

Fig. 6. EDX spectrum for p(HEMA-MAGA) with 10 wt.% in feed (Ca/P=1.75).

5. Conclusions

This article proposed the obtaining of new biomaterials that can be used for bone reconstruction systems. We have synthesized 2-hydroxyethyl methacrylate – 2-methacryloylamdio glutamic acid copolymers with different compositions and we evaluated their ability to initiate the formation of HA crystals *in vitro*. The results were very encouraging and SEM analysis showed the presence of calcium and phosphorus onto the surfaces of all materials and the molar Ca/P ratio evaluated by EDX proved the presence of apatite-like crystals (values close to 1.67).

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