Crystallization patterns of calcium phosphate precipitated on acrylic hydrogels in abiogenic conditions

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The process of precipitation of sparingly soluble calcium salts has attracted much attention both from those investigating the role of this phenomenon in industrial applications (e.g. wastewater treatment, desalination, sedimentation, scale formation on heat exchanger surfaces, etc.) and from those attempting to simulate *in vitro* the biogenic formation of mineral phases through normal calcification (enamel, dentin, cementum and bone) or pathologic calcification (calculi, stones, arteriosclerotic plaques, arthropathic deposits, dental plaques, etc.). Yet, the process is not completely understood even in the case of the ubiquitous calcium phosphate phases, commonly idealized as hydroxyapatite. Classic studies [1–3] indicate that the precipitates formed immediately upon mixing pure solutions of calcium and phosphate salts possess a fine, amorphous texture which is metastable with respect to apatites. It was inferred [1] that newly formed bone and enamel may contain similar amorphous components and it is accepted now that amorphous calcium phosphate phases play a role in biogenic calcification [4]. While biogenic calcification leads to well-defined, highly organized mineral phases (e.g. bone or enamel) due to a high level of molecular control, in abiogenic conditions that involve the rapid mixing of solutions of calcium and phosphate salts, completely unstructured precipitates are generated as the kinetically driven process is fast and little controlled. These amorphous phases are unstable and it is generally accepted that they undergo conversion into more ordered and thermodynamically favoured crystalline phases such as octacalcium phosphate or hydroxyapatite [1, 3–6], although this presumption has been challenged recently [7]. Aiming at a higher reproducibility of the biogenic conditions *in vitro*, methods have been developed where the composition of solution was maintained constant [8, 9] or, additionally, the rate of crystallization was slowed down by restraining the diffusion [10, 11]. Typical hydroxyapatite morphologies were observed in such experiments immediately after precipitation, suggesting the suppression of transient amorphous phases.

In addition to precipitation of calcium phosphate phases in solution, the crystalline growth and epitaxy on solid substrates are also of relevance to the understanding of hydroxyapatite formation and biogenic calcification [12]. The evidence from biomineralization studies suggested that the organized organic surfaces of the biopolymers serving as substrates can control the nucleation and growth through specific molecular processes involving chemical, stereochemical, electrostatic and geometric (epitaxial) interactions and complementarities [13]. It was further surmised [14] that biomineralization proceeds through the stages of supramolecular pre-organization (self-assembly of the substrate), interfacial molecular recognition (templating) and cellular processing. However, biopolymers are not the only materials to raise interest as substrates for calcium salts deposition. The advent of cardiovascular and ophthalmic polymeric biomaterials, of which a major drawback is their dystrophic calcification, has emphasized the need to also elucidate the role of synthetic polymers as substrates and their effect on nucleation and growth. As most of the synthetic biomaterials are structurally much simpler than the biopolymer substrates, their calcification in biogenic and, especially, abiogenic conditions is expected to proceed through less complicated pathways, although chemical, stereochemical and electrostatic interactions must still be involved, together with some sort of molecular recognition through a match

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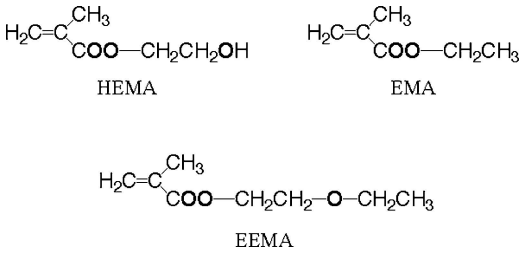

Figure 1 Chemical structure of the monomers used to make the hydrogels.

between functional groups on the polymer and the precipitating ions. For instance, in two series of synthetic acrylic polymers, the results of experimental *in vivo* calcification (animals) [15] or *in vitro* crystallization of calcium oxalate [16] showed an obvious sensitivity of each process to differences in the chemical structure of polymers.

Here, we pursue our quest to understand the mechanism of spontaneous abiogenic calcification of synthetic hydrogels based on 2-hydroxyethyl methacrylate (HEMA), after we have discussed previously a number of potential scenarios [17] and have subsequently demonstrated [18] the plausibility of a hypothesized local enhancement of supersaturation within the polymer network due to salting-out ionic solute effects. The chelation of calcium ions to the polar groups of the substrate, leading to calcium ion complexes that can serve as initial nucleation sites, is a hypothesis previously employed in the context of biogenic calcification [19–21] and confirmed *in vitro* in particular cases [22]. If valid, it would account both for templating at the polymer interface and for the sensitivity of the calcium deposition to changes in the polymer's chemical structure. We wish to extend this hypothesis to the abiogenic calcification of HEMA-based polymers. Poly(2-hydroxyethyl methacrylate) (PHEMA) contains, in every monomeric structural unit, three polarizable oxygen atoms to which calcium ions have a great tendency to chelate. Changing the oxygen content or the chemical environment around the oxygen atoms by modifying the structure of the polymer would result in changes in both crystallization patterns and/or the amount of calcium deposited. If such effects are observed in a predictable fashion, such evidence may support the chelation theory.

We synthesized PHEMA and two series of copolymers of HEMA, one with ethyl methacrylate (EMA) and another with 2-ethoxyethyl methacrylate (EEMA) (Fig. 1). Each series consisted of two copolymers containing respectively 10% and 20% (by weight) of each comonomer. While in the HEMA/EMA copolymers the oxygen content available for chelation decreases due to less oxygen atoms per structural unit, in HEMA/EEMA copolymers the oxygen content decreases globally due to larger amount of carbon relative to oxygen. Sheets of polymers were prepared by polymerizing HEMA or HEMA/comonomers mixtures, in the presence of 0.35% ethylene dimethacrylate as a crosslinking agent and 0.1% 2,2-azo-*bis*-(2,4-dimethyl valeronitrile) (V-65®) as an initiator, between glass plates spaced at 2 mm, at 50 °C (oven) for 24 hr. The plates were lined with transparency films (CG6000, 3M Co.) to facilitate the removal of resulting sheets. After curing the sheets at 100 °C for 3 hr, they were extracted for 2 days in a Soxhlet extractor and disks of 7-mm diameter were cut and stored in sterile water of zero osmolality (Viaflex®, Baxter). For the calcification experiments, solutions of $CaCl_2$ (2.57×10^{-3} mol L^{-1}) and Na_3PO_4 (1.54×10^{-3} mol l^{-1}) were prepared. After adjusting the pH in the latter to 8 with HCl, equal volumes of each solution were mixed to provide a metastable calcifying medium with a ratio $[Ca^{2+}]/[PO_4^{3-}]=1.67$ (as in hydroxyapatite) and pH 7.3. To study the crystallization patterns, one disk of each polymer was immersed in a vial containing 3 ml of the calcifying medium and incubated at 37 °C in a shaker at 150 cycles/min for 2 weeks. The disks, which all presented white deposits, were examined in a variable pressure scanning electron microscope (Zeiss/Leo 1555FEG SUPRA 55VP). For the quantitative determination of the calcium content, five disks of each polymer were stored in the calcifying medium for 6 weeks at 37 °C in static conditions. The medium was replaced twice a week with fresh solution. The calcified disks were ashed at 600 °C in platinum crucibles, digested with HCl, and the resulting solutions were analyzed by inductively coupled plasma emission spectroscopy in a Vista PRO CCD Simultaneous ICP-OES (Varian) instrument using calcium emission lines at five different wavelengths and averaging the results.

The SEM photographs of the precipitates are shown in Fig. 2. The crystals deposited on PHEMA have a spherical aspect (Fig. 2a), confirming our previous observations [23]. Precipitation of calcium phases in solution as spherules is traditionally related to amorphous components [3, 4], although ordered phases can adopt similar morphologies [10, 11, 24]. Deposition of spherical agglomerates has been reported on synthetic polymers [11, 16], cellulose [24], glasses and metal oxide gels [25] and on cardiovascular tissue and prostheses [26]. The precipitates that we observed on the two copolymer substrates had a different morphology from those on PHEMA, but were indistinguishable between them (Figs. 2b and c). These deposits consist of particles aggregated loosely into irregularly shaped, flocculent clusters. Such morphology was seen in amorphous species before they underwent fast changes (within minutes) [1, 4]. In comparison, our samples are considerably aged.

The absolute quantitation of the calcium incorporated by polymers provided unexpected results (Table I). Copolymerization of HEMA with EMA, despite leading to materials with less chelation capacity, did not cause less precipitation. On the contrary, both copolymers HEMA/EMA retained more deposited calcium than the homopolymer. Surprisingly, calcification was much lower in the HEMA/EEMA copolymers (Table I), although the decrease in chelation capacity was relatively minor in these materials. The results in Table I do not endorse the chelation of calcium ions as a source for nucleation sites. Alternatively, the differences in calcium deposition on the three

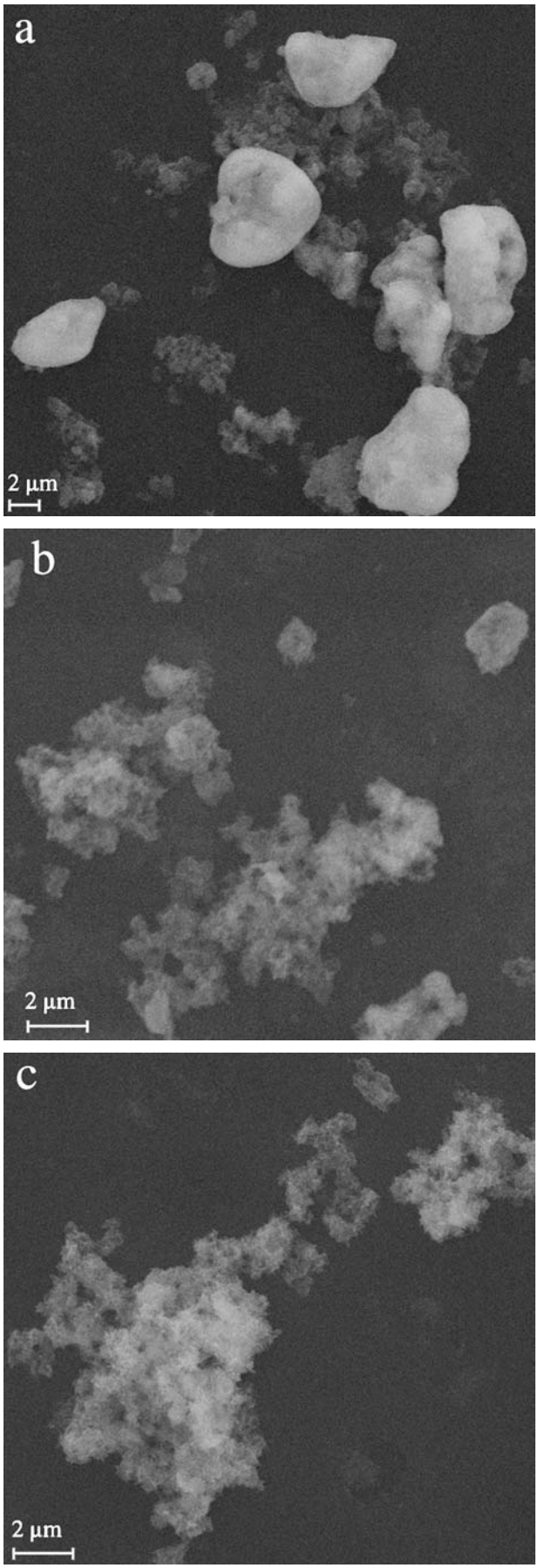

Figure 2 Scanning electron micrographs of the surface of calcified polymers: (a) PHEMA; (b) copolymer HEMA (90%) – EMA (10%); (c) copolymer HEMA (80%) – EEMA (20%). Instrument: VPSEM; accelerating voltage: 15 kV; chamber pressure: 31 Pa.

TABLE I Calcium content in the calcified polymers determined by ICP.

Substrate[a]	Calcium content[b] (mg calcium/g polymer)
PHEMA	7.0 ± 1.1
P(HEMA-*co*-EMA) (90:10)	11.9 ± 0.6
P(HEMA-*co*-EMA) (80:20)	8.2 ± 0.5
P(HEMA-*co*-EEMA) (90:10)	2.8 ± 0.7
P(HEMA-*co*-EEMA) (80:20)	1.4 ± 0.4

[a]HEMA: 2-hydroxyethyl methacrylate; EMA: ethyl methacrylate; EEMA: 2-ethoxyethyl methacrylate; PHEMA: poly(2-hydroxyethyl methacrylate); "*co*" denotes copolymers.
[b]Given as mean values ± standard deviation ($N = 5$), rounded to one decimal place

polymers reflect structural factors and surface characteristics that outweigh the changes in their chelation capacity.

To conclude, in our attempts to confirm a role for the chelation of calcium ions in the calcification of synthetic hydrogels, we unveiled here an intricate correlation between the chemical structure of polymers and their propensity for calcifying. This correlation predominates over the possible effects of changing the chelation capacity and may rely upon stereochemical complementarities involving polymer structural features and calcium ions, as well as upon surface energetics. The abiogenic calcification on synthetic polymers may be, therefore, as complex as that suggested for the biogenic substrates.

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