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Structure and biological response of polymer/silica nanocomposites prepared by sol-gel technique

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Abstract

Structure of P(EMA-co-HEA)/SiO₂ nanocomposites with silica content in the range from 0 to 30 %wt was correlated with cell behaviour on substrates of those compositions by making use of two different populations of primary human cells: articular cartilage chondrocytes and dental pulp cells. Substrates were prepared by the simultaneous copolymerization of the organic monomers and the sol-gel reaction of the silica precursor in different proportions, which led to weight fractions of the silica phase in the materials closely matching the stoichiometric ratios employed during the preparation, both in the bulk and at the material surface. The silica nanophase increases surface wettability and improves the mechanical properties of the base materials. Both chondrocytes and dental pulp cells were cultured on serum-coated nanocomposite substrates in the same conditions, but very different cellular responses were obtained. While chondrocytes adhered and proliferated, dental pulp cells formed viable aggregates weakly adhered on the sample that were viable up to 11 days. The results suggest that these sol-gel derived nanocomposites may be used as culture surfaces maintaining the dental pulp cell phenotype in vitro.

Keywords: Nanocomposite, hybrid, acrylate, silica, cellular response

Introduction

Polymer hydrogels stand out among synthetic materials used as biomaterials because of their good biocompatibility due, mainly, to their ability to absorb large amounts of water. However, they have poor mechanical properties and, as a consequence, they are in need of reinforcement. There are several routes to reinforce a polymer hydrogel, including the copolymerization with a hydrophobic component and the preparation of interpenetrating polymer networks [1,2]. A different strategy consists in the preparation of hybrid organic/inorganic nanocomposites, which include a silica phase obtained simultaneously with the polymer in a sol-gel process. [3].

Apart from reinforcement, there is a great need of providing bioactivity to surfaces in applications where good interaction between the synthetic material and the living tissues is mandatory. Since the discovery of the 45S5 Bioglass® by Hench in 1971 [4], which appeared to strongly adhere to bone tissue, various kinds of ceramics such as Na₂O-CaO-SiO₂-P₂O₅ glasses, sintered hydroxyapatite and glass-ceramics containing apatite or wollastonite, are known to bond directly to living bone avoiding the formation of fibrous tissue [5-7]. Some of them or their derivatives are being clinically used in artificial middle-ear bone implants, artificial vertebrae, intervertebral discs, iliac bones, fillers in bone and maxillofacial defects or to fill the gaps around the implants, etc. and have inspired new bioactive materials with varied compositions and structures to fulfill different applications [8-13]. However, the use of bioactive glasses for the stimulated regeneration of tissues is limited by their poor mechanical properties, since their brittleness makes difficult their use in load-bearing applications.

One approach to combine bioactivity and enhanced mechanical properties in the same material is the preparation of organic/inorganic hybrids, in which the inorganic phase is incorporated into an organic polymeric matrix. This type of structural organization to a certain extent mimics the composition of mineralized tissues, where the hydroxyapatite

(HAp) inorganic phase is dispersed in a collagen matrix with which it interacts at a molecular level yielding a natural composite. Various hybrid systems have been explored employing as inorganic phases bioactive glasses [15,16], HAp [17-19] or pure silica [25], in the form of particles or fibres. Porous scaffolds of hybrid composites mimicking natural bone structures have been prepared and investigated to serve as a support and guide new tissue in-growth and regeneration [20-23]. In the recent past many studies have been devoted to organic/inorganic nanocomposites with the aim of combining the properties of both phases at the nanoscale level. Polymer/silica nanocomposites have been prepared by physically mixing silica nanoparticles with polymers [24,25] or by copolymerization of the organic polymers with surface-functionalized silica nanoparticles [26,27]. Various surface modification techniques have also been proposed aiming to enhance the bioactivity of polymer surfaces. These methods are based on the introduction of hydrophilic polar groups, such as phosphate, hydroxy groups, Si-OH or Ti-OH, and carboxy and carboxylate groups, which have been found to be effective for apatite nucleation, onto more hydrophobic less bioactive substrates [28-30]. Nonetheless, the idea of nanohybridizing biocompatible polymers with silica by this simultaneous *in situ* sol-gel polymerization to improve their bioactivity is a very recent one. Few published works address it. Rhee *et al.* [28,29] polymerized poly(ϵ -caprolactone) simultaneously via sol-gel with tetraethoxysilane (TEOS) as silica precursor and calcium nitrate tetrahydrate to dope the nanohybrid with calcium ions to facilitate the apatite nucleation. Costa *et al.* [30] polymerized poly(2-hydroxyethyl methacrylate) by the same procedure with tetramethoxysilane (TMOS) as silica precursor and characterized the hydroxyapatite layer formed.

In previous works we addressed the structure, physicochemical properties and hydroxyapatite nucleation ability of poly(ethyl methacrylate-*co*-hydroxyethyl

acrylate)/silica, P(EMA-*co*-HEA)/SiO₂, nanohybrids with silica contents comprised between 0 and 30 %wt as potential candidates for the synthetic matrix of scaffolds for bone or dentin regeneration [3,31,32]. Here we analyze the effect of the presence of silica on the *in vitro* biological performance of this series of substrates making use of two different human primary cells: articular cartilage chondrocytes and dental pulp cells. This work is a first attempt to understand the interaction of these specific sol-gel derived nanocomposites with human cells *in vitro*. We employed two cell populations stemming from human tissues with very different properties and lineage. Chondrocytes are mature cells which are in need of adequate substrates for their *in vitro* expansion while maintaining their phenotype; dental pulp cells are immature, multipotent cells able to differentiate to osteoblasts. Our results show that sol-gel derived nanocomposites might be useful for maintaining the characteristic dental pulp phenotype *in vitro*, minimizing interactions with the artificial substrate while keeping the cell population viable.

Materials and methods

Synthesis of P(EMA-co-HEA)/SiO₂ nanohybrids

Nanocomposites of poly(ethyl methacrylate-*co*-hydroxyethyl acrylate), p(EMA-*co*-HEA), with fixed EMA/HEA weight ratio of 70/30 %wt and with varying proportions of silica, SiO₂: 0, 5, 10, 15, 20 and 30 %wt, were obtained in the form of sheets of 0.8 mm in thickness similarly as in [3]. Briefly, the procedure consisted in preparing an organic mixture of the organic monomers ethyl methacrylate, EMA (99%, Aldrich), and hydroxyethyl acrylate, HEA (96%, Aldrich), with a 0.5 %wt of ethylene glycol dimethacrylate, EGDMA (98%, Aldrich), as crosslinking agent and a 2 %wt of benzoyl peroxide, BPO (97%, Fluka), as thermal initiator, relative to monomer weight.

Separately, tetraethoxysilane, TEOS, was mixed with distilled water and hydrochloric acid (37%, Aldrich) in the molar ratio 1:2:0.0185, respectively (inorganic mixture). After 30 min of separate stirring, both solutions were mixed, stirred for another 30 min and injected into glass moulds. The hybrid mixture was polymerized in an oven at 60°C for 21 h and post-polymerized at 90°C for 18 h, rinsed in boiling distilled water/ethanol mixture 50/50 %vol for 24 hours to eliminate monomer residues and finally dried in a vacuum desiccator at 80°C until constant weight. Materials with silica contents of 0, 5, 10, 15, 20 and 30 %wt were obtained by calculating the (EMA+HEA)/TEOS ratios assuming that the sol-gel reaction was complete. Hereafter, the hybrids will be referred to as H_x, x being the percentage of silica. Besides, poly(ethyl methacrylate), PEMA, and poly(hydroxyethyl acrylate), PHEA, homopolymers were prepared following the same procedure as reference systems.

Fourier-transform infrared spectroscopy (FTIR) analyses

Fourier-transform infrared (FTIR) spectra were collected in a Thermo Nicolet Nexus FTIR spectrometer (Thermo Fischer Scientific Inc., Waltham, MA, USA), in the attenuated total reflection mode (ATR), to elucidate the surface compositions. The spectra resulted from averages of 128 scans at 4 cm⁻¹ resolution, between 650 and 4000 cm⁻¹.

Quantification of SiO₂ by energy dispersive x-ray spectroscopy (EDS)

The percentage of silica at the surfaces and in the interior of the nanohybrids was quantified by energy dispersive x-ray spectroscopy (EDS) in an Oxford Instruments spectrometer, attached to a JSM-6300 scanning electron microscope (JEOL Ltd.,

Tokyo, Japan). Exterior and inner (fracture) surfaces were analyzed. Samples were previously sputter-coated with carbon under vacuum. Spectra were taken at 10 kV of acceleration voltage and 15 mm of working distance. Silicon was employed as optimization standard.

Contact angle measurements

In order to determine the surface wettability of the different substrates, contact angle measurements were conducted in air atmosphere with the sessile drop method, employing water (extra pure, Scharlau) as drop liquid. A Dataphysics OCA instrument (DataPhysics Instruments GmbH, Filderstadt, Germany) was used for this purpose. Glass Pasteur pipettes were employed as droppers. A minimum of 10 drops of water was analyzed for each composition.

Compression tests

Mechanical compression tests were performed in a Seiko TMA/SS6000 device (Seiko Instruments Inc., Chiba, Japan), from 0.05 to 150 g at 10 gmin^{-1} , at room temperature. Specimens were disk-shaped (3 mm in diameter, 0.8 mm-thick) and each composition set consisted of five replicas.

Chondrocytes

Human articular cartilage from the knee of a patient undergoing total knee arthroplasty was processed for chondrocyte isolation. Briefly, the cartilage tissue was aseptically dissected from the joint, minced, and washed with Dulbecco's modified Eagle's medium (DMEM, Life Technologies). Then, the cartilage was incubated for 30 min with a 0.5 mg/mL hyaluronidase (Sigma-Aldrich) solution and for 1 h with a 1 mg/mL

pronase (Merck) solution in a shaking water bath at 37°C. After that, the cartilage fragments were washed with DMEM and incubated with a 0.5 mg/mL collagenase-IA (Sigma-Aldrich) solution in a shaking water bath at 37°C overnight. The resulting cell suspension was filtered with a 70 µm cell strainer (BD Biosciences) to remove any undigested tissue and collagenase was rinsed off with DMEM containing 10% FBS (Invitrogen SA). Finally, the cell suspension obtained was transferred in 15 mL DMEM supplemented with 10% FBS and 50 µg/mL ascorbic acid (Sigma-Aldrich) to a 75 cm² tissue culture flask (Nunc) and maintained at 37°C, in a humidified atmosphere under 5% CO₂. The culture medium was replaced every 2 days and cells were allowed to grow until subconfluence. Then, the cells were harvested by trypsinisation and counted with a hemacytometer for the experiment.

Dental pulp cells

Human dental pulp cells were isolated from third molar teeth extracted due to odontological reasons from healthy patients. First, pulp was extracted and washed with Hank's balanced solution (Gibco-Invitrogen, USA) supplemented with penicillin/streptomycin (100 U/ml and 100 µg/ml respectively, Gibco-Invitrogen). Pulp was minced in small fragments and incubated with a 2 mg/mL collagenase-IA (Sigma-Aldrich) solution in a shaking water bath at 37°C for 90 min. Cellular suspension was centrifuged (400 g for 7 min) and the resulting pellet was seeded on a 75 cm² tissue culture flask (Nunc) in DMEM containing 10% FBS and antibiotics and maintained at 37°C, in a humidified atmosphere under 5% CO₂. The culture medium was replaced every 2 days and cells were allowed to grow until subconfluence. Then, the cells were harvested by trypsinisation and counted with a hemacytometer for the experiment.

Cell culture

Films pre-sterilized with 25 kGy gamma radiation were placed in a 96-well tissue culture plate and were soaked in culture medium for 72 h before cell seeding. Then, 40 μL (10^4 cells) of the cell dispersion were placed onto the polymer films and were incubated at 37°C under 5% CO_2 condition for 1 h. After this time, 600 μL of fresh medium were added to each well. Samples were maintained at 37°C , in a humidified atmosphere under 5% CO_2 for 10 days. The culture medium used was DMEM supplemented with 10% FBS and 50 $\mu\text{g/mL}$ ascorbic acid and it was renewed every 2–3 days. Contrast phase light microscopy was carried out to observe morphological changes in cell shape after different time cultures. Neutral red staining was employed as a vital staining. Each experiment was performed in triplicate and TCPS served as the control substrate.

Results and discussion

Figure 1 shows the FTIR spectra of the nanocomposites and both homopolymers. The CH_x asymmetric and symmetric stretching peaks appear at 2962 and 2888 cm^{-1} , respectively, in all spectra, and the broad band between 3700 and 3100 cm^{-1} , as well as the shoulder at 1650 cm^{-1} , characteristic of the hydroxy groups, only appears in the PHEA spectrum (data not shown). The well-defined C=O stretching peak (1700 cm^{-1}) decreases with the percentage of silica. Between 1700 and 650 cm^{-1} the FTIR curves show the complex spectrum of the copolymer blurring the silica fingerprint region. The intensity of the peaks at 1100 cm^{-1} and 800 cm^{-1} (Si-O-Si asymmetric and symmetric stretching, respectively) and 950 cm^{-1} (Si-OH stretching), characteristic of the silica phase [28,29], increases with the silica content. No evidence of hybrid Si-O-C bonds from heterocondensation reactions can be found by FTIR, because their characteristic

peak ($1120\text{-}1080$ and 836 cm^{-1}) [29] overlaps the absorption interval of the Si-O-Si bonds. The absence of the broad band characteristic of the OH groups of the silanols or the OH vibrations of HEA can be explained by an imperfect contact of the sample and the measuring device, caused by the rigidity of the samples at room temperature. This band appeared only after immersion of the samples in water to render them softer (data not shown).

The experimental silica contents, SiO_2 EDS, listed in Table 1 were calculated from the silicon average contents obtained by EDS, making use of the silica and silicon molar masses and neglecting the amount of hydrogen in the copolymer and in the silanol groups, undetectable by this technique and the carbon coating of the samples for performing the EDS measurements. The surface and bulk silica percentages are very similar and correlate quite well with the nominal silica contents of the hybrids, despite the considerations concerning the detection of hydrogen and carbon. This is an important issue since the biological media will interact with the components on the surface of the sample. The larger standard deviations of the silica contents obtained in the bulk are due to the limitations of this technique when working with fractured samples of variable height. Both the FTIR spectra and the silica quantifications by EDS corroborate that TEOS was efficiently hydrolyzed and condensed to silica during the sol-gel process, and silica was found to be homogeneously distributed throughout the polymeric matrix both in the bulk and at the surface.

The average water contact angles, θ_w , are also listed in Table 1. The water contact angle of the H05 surface is identical to that of the copolymer, lying between those of the homopolymers. It then decreases for intermediate silica contents (10-20 %wt) and increases again for the H30 surface, reaching a value close to that of the copolymer. The compressive elastic moduli, E , were obtained from the initial slopes of the stress-strain

curves, and are also listed in Table 1. The modulus of the copolymer lies between those of the homopolymers, and maintains similar values up to 15 %wt of silica, from where it starts increasing up to a value similar to that of PEMA for the H30 composite.

As explained in a previous work [31], different inorganic phase morphologies are obtained within the copolymeric matrix depending on the silica content. Below a 15 %wt of silica the inorganic phase is uniformly dispersed in the nanocomposites as porous nanoparticles which form larger aggregates leaving spaces of tens of nanometers. Higher silica contents produce silica networks which percolate and eventually extend continuously throughout the samples, totally interpenetrated with the organic network. These different phase morphologies influence the physicochemical properties of the surfaces. The number of free Si-OH groups is larger the larger the boundary interface of the silica phase, and thus decreases as percolation occurs and with the development of the continuous silica network. This is reflected in the wettability of the nanocomposites with high silica contents. Besides, the reinforcing effect of the interpenetrated silica phase is observed in the increase of the compressive elastic modulus of the nanohybrids.

Cells respond to different kinds of surface parameters: chemical, topographical and mechanical, through the intermediacy of extracellular matrix (ECM) proteins. Here we investigated the role of increasing amounts of silica of the material surface on the biological performance of the substrate. Cellular behavior at the biomaterials interface can be understood in terms of the natural adhesive interaction of cells with the ECM [33]. Cells do not interact directly with foreign materials, but they attach to the adsorbed layer of proteins on the material surface, such as fibronectin (FN), vitronectin (VN), fibrinogen (FNG), representing the so called soluble matrix proteins in the biological fluids [33]. Cells recognize these matrix proteins via integrins - a family of cell surface

receptors – that provide trans-membrane links between the ECM and the actin cytoskeleton [34]. When integrins are occupied they cluster and develop focal adhesion complexes that actually anchor the cells to the surface and trigger the subsequent cellular response [34]. Thus, the cell-material interaction is a complex process consisting of early events, such as adsorption of proteins, followed by cell adhesion and spreading, and later events, related to cell growth, differentiation, matrix deposition and cell function.

Figure 2 shows human articular chondrocytes after 1 day of culture on the different substrates. Cells are well spread and distributed throughout the surface of the samples independently of the amount of silica in the material (from 5 to 30 %). The fraction of the surface covered by cells is lower for the homopolymers (both PEMA and PHEA) than for the 70/30 %wt copolymer. This family of polymers represents a set of materials with very different mechanical properties (see table 1) that support chondrocyte expansion. From an applied perspective, the incorporation of silica within the polymer matrix represents a way of matching the properties of the material to those of articular cartilage; since the stiffness of the sample is known to influence the cell-material interaction [35-37], it is important in our case that this increased stiffness does not modify the biological performance of the substrate. Chondrocytes remain covering the surface of the material and start growing in layers (cell on cell) up to 10 days on culture (results not shown, microscopy images are similar to those shown in Figure 2 where a confluence monolayer is already formed).

The situation is completely different when the same family of materials is seeded with human dental pulp cells (Figure 3). After two days of culture the cells are spread on PEMA, PHEA and the 70/30 %wt copolymer. However, addition of silica to the system leads to a complete modification of the cell-material interaction, which changes

dramatically as the amount of silica in the system increases. Cells tend to form clusters which adhere weakly to the surface of the material, in a growing number as the silica content increases. This is in agreement with the results found by Cousins *et al.* [38] with L929 murine fibroblasts seeded on silica nanoparticulate coatings, which showed a weakly adhered rounded conformation in comparison with the same cells seeded on a plain glass surface, with spread flattened morphology. These authors hypothesized that the nanostructure of silica governs the serum proteins-substrate interactions, leading to localized focal adhesion sites, and thus to weakly adhered rounded cells. In our case, the presence of silica on the material substrate implies the presence of a higher concentration of OH groups on the material and a slightly negatively charged surface that leads to a more wettable surface (table 1).

After 5 days of culture the cells remained clustered on the compositions with the lower content of silica (5, 10 and 15 %wt) while good adhesion and proliferation occurred on the samples with 20 and 30 %wt silica (Figure 4). This behavior was previously found on TEOS based chitosan/silica membranes seeded with fibroblast-like cells. Cell growth rate, density and adhesion strength were lower for hybrid materials than for pure chitosan for lower silica contents (below 20 %), but similar or even increased values were obtained for a 50 %wt inorganic content [39].

The pulp cells are shown to be viable after 5 days on the material as revealed by neutral red staining for the cell clusters (Figure 5). Besides, when these cells are collected from the nanocomposites and seeded again on a standard substrate they acquire the typical fibroblast-like morphology (Figure 5). This peculiar cell-material interaction behavior has interesting consequences for tissue engineering applications requiring phenotype maintenance.

It is interesting to stress that the same nanocomposite materials, coated with the same solution of proteins (FBS in our case, that consists mainly of fibronectin and vitronectin), has a very different cell response depending on the cell type seeded, which reveals the specificity of the cell-material interaction. It is known that human chondrocytes tend to spread on material surfaces and adopt an extended morphology as a consequence of the production of collagen and expression of the $\alpha_2\beta_1$ integrin that mediates interaction with this protein [40]. However, dental pulp cells are in need of fibronectin and vitronectin adsorption on this set of substrates to adhere via the $\alpha_5\beta_1$ or $\alpha_v\beta_3$ receptors [41] which does not happen when silica is dispersed on the material surface due to conformational changes of fibronectin after adsorption on silica, reducing the availability of cell-binding domains [42].

Conclusions

Polymer/silica nanocomposites were prepared following a sol-gel process that leads to a material morphology of interpenetrated phases with controlled known concentration of silica both in the bulk and at the surface of the hybrid system. The incorporation of silica improves the mechanical properties of the polymer system and may influence the cellular response. Chondrocytes and dental pulp cells are both viable on the hybrid surfaces, but major differences are found between both cell types: chondrocytes are able to adhere and proliferate independently of the silica content of the surface, whereas dental pulp cells adhere only weakly and form clusters of cells that remain stable and viable up to 11 days.

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Captions to Figures

Figure 1. FTIR spectra of the different samples, in the 650-1850 cm^{-1} region.

Figure 2. Phase light contrast microscopy images of human articular chondrocytes on the different substrates after 1 day of culture. (a) PEMA, (b) PHEA, (c) P(EMA-co-HEA) 70/30 %wt copolymer. Nanohybrids with increasing amount of SiO_2 (d) 5%, (e) 10%, (f) 15 %, (h) 20%, (i) 30%. Last image (j) represents control TCPS.

Figure 3. Phase light contrast microscopy images of dental pulp cells on the different substrates after 2 day of culture. (a) PEMA, (b) PHEA, (c) P(EMA-co-HEA) 70/30 %wt copolymer. Nanohybrids with increasing amount of SiO_2 (d) 5%, (e) 10%, (f) 15 %, (h) 20%, (i) 30%. Last image (j) represents control TCPS.

Figure 4. Phase light contrast microscopy images of pulp cells on the different substrates after 5 day of culture. (a) PEMA, (b) PHEA, (c) P(EMA-co-HEA) 70/30 %wt copolymer. Nanohybrids with increasing amount of SiO_2 (d) 5%, (e) 10%, (f) 15 %, (h) 20%, (i) 30%. Last image (j) represents control TCPS.

Figure 5. Phase light contrast microscopy images of human dental pulp cells on the nanocomposites. (left) 10 culture days on TCPS after culture for 4 days on the substrate with 15 %wt SiO_2 . (right) Neutral red staining shows cell viability on H05 after 8 days.

Table 1. Average SiO_2 contents obtained by EDS, SiO_2 EDS, on the surface and in the bulk, water contact angles, θ_w , and compressive elastic moduli, E .

Sample	SiO_2 EDS (%)		θ_w (°)	E (GPa)
	surface	bulk		
PEMA	-	-	82.6±1.6	4.2±0.1
PHEA	-	-	96.1±3.9	0.14±0.03
H00	-	-	91.8±2.3	2.6±0.2
H05	5.18±1.06	5.60±4.00	92.0±1.6	2.7±0.3
H10	9.06±0.21	8.42±0.61	80.1±2.9	2.8±0.3
H15	15.09±0.27	15.33±3.12	76.5±1.4	2.9±0.1
H20	21.21±0.61	22.51±0.95	76.8±4.1	3.3±0.2
H30	28.45±0.58	24.87±3.60	90.5±2.2	3.9±0.4

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5