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Hydroxyapatite containing superporous hydrogel composites: synthesis and in-vitro characterization

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Abstract The synthesis of an acrylamide-based superporous hydrogel composite (SPHC) with hydroxyapatite (HA) was realized by solution polymerization technique. The characterization studies were performed by FTIR studies, determination of swelling kinetics, measurement of mechanical properties, SEM/EDAX studies and cytocompatibility tests. The FTIR and EDAX studies revealed the incorporation of HA in superporous hydrogel (SPH) structure. The results obtained from swelling experiments showed that, although the extent of swelling was decreased after incorporation of HA in SPH structure, the time to reach the equilibrium swelling was not affected for SPHC. This result indicated that, the presence of HA did not block the capillary channels and the interconnected pore structure was maintained which were consistent with the images obtained from SEM photographs. The results obtained from mechanical tests showed that, in the presence of HA, the compression strength of the hydrogel composite was improved significantly when compared to SPH structure. The compressive modulus for the SPHC increased to 6.59 ± 0.35 N/mm^2 whereas it was 0.63 ± 0.04 N/mm^2 for the SPH. The cytocompatibility test which was performed by using L929 fibroblasts showed that both the SPH and SPHC materials were cytocompatible towards fibroblasts. The synthesized superporous hydrogel composite possesses suitable properties especially for bone tissue engineering applications and shall be considered as a novel scaffold.

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Introduction

There has been a significant advance in the use of hydrogels as tissue engineering scaffolds. In their aqueous environment, the pores large enough will let the diffusion of nutrients/wastes and accommodate the living cells [1]. In recent studies, hydrogels with fast swelling property have been synthesized by using a gas blowing technique. These hydrogels having interconnected pores, of which diameters are in the order of a few hundred micrometers are called 'superporous hydrogels' (SPHs) [2]. With their large pore size and high swelling ratio, SPHs have many advantageous as a tissue engineering matrix, like the diffusion of nutrients, metabolites, growth factors and penetration and proliferation of living cells. However, a significant disadvantage is their low mechanical strength due to their superabsorbent nature. A recent approach to gain increased mechanical properties is to synthesize SPH composites (SPHCs) by adding different materials [3]. The synthesis and the structure of SPH and SPHCs have been explained by Chen et al. [2, 3]. Furthermore, Doorkosh et al. have designed novel drug delivery systems for oral administration of peptide and protein based drugs using SPH and SPHC as the carrier [4–6].

In the biomedical field, the synthesis of hydroxyapatite (HA)/polymer composite materials is of great interest for the development of biomaterials especially suitable to repair the skeletal system [7, 8]. HA, $Ca_{10}(PO_4)_6(OH)_2$, is the main inorganic component of human bones, possessing significant bioactive and osteoconductive properties [9]. It is considered as an appropriate reinforcement for organic polymers for the possible use as bone cements [10], dental implants [11] and bone replacement materials [12, 13]. HA/polymer composites have also been attempted in hard tissue engineering for the substitution of large bone defects

[14]. The advantages of HA composite materials are reported to be the possibility to modulate bioactivity and mechanical properties with an improved interfacial bonding of composite with bone tissue [15].

In this study, we aimed to synthesize superporous hydrogel composites with HA for increased mechanical strength and bioactivity. SPHC was prepared by adding HA beads of size between 25–45 μm to the monomeric mixture and polymerization was realized in the presence of HA. The incorporation of HA particles to SPH structure was evaluated by FTIR and EDAX analysis. The physical properties like swelling behavior, density and void fraction were investigated by using suitable methods. The increase in mechanical properties was evaluated by compression tests. The size and interconnectivity of pores were examined under SEM. The cytocompatibility of synthesized hydrogel composites was also investigated for their possible use in tissue engineering applications.

Materials and methods

Materials

Acrylamide (AAm) was purchased from Across Organics (USA). HA with particle size between 25–45 μm (determined by the manufacturer) and ammonium persulfate (APS) were obtained from Fisher Scientific Company (USA). Acrylic acid (AAc) was obtained from Aldrich (USA). *N*,*N*,*N*′,*N*′-tetramethylene diamine (TEMED), sodium bicarbonate ($NaHCO_3$), *N*,*N*′ methylenebisacrylamide (Bis), Pluronic® F127 (PF127), glutaraldehyde and hexamethyldisilazane were purchased from Sigma Co. (Germany).

Synthesis of superporous hydrogels

Superporous hydrogels and hydrogel composites were synthesized as described by Chen et al. [2, 3]. The following components were added sequentially into 16 × 100 mm glass test tube at ambient temperature: 1,000 μL of AAm, 70 μL of 2.5% Bis, 460 μL of distilled water, 30 μL of PF 127 and 45 μL of AAc, 40 μL of 20% APS and 40 μL of 20% TEMED. The test tube was extensively mixed after adding each component. After 300 s 90 mg $NaHCO_3$ was added to the mixture, and the tube was vortexed. Polymerization was allowed to continue for 1 h at room temperature. The synthesis of SPHCs was similar to SPH. HA was added to the monomer mixture after adding APS and before adding TEMED. Three different amounts of HA particles, i.e. 250, 1,000 and 2,000 mg were used to determine their effects on composite structure and properties. Table 1 shows the receipt used in our study. The synthesized hydrogels were removed from the tube and allowed to swell in water before drying in order to remove the unreacted components. The swollen hydrogels were dehydrated in 10 mL absolute ethanol followed by a further dehydration step in 50 mL absolute ethanol. After this procedure the hydrogels were removed from ethanol and dried at 60 °C overnight.

FTIR spectroscopy

The chemical structure of the synthesized hydrogels were investigated by using Fourier Transform Infrared spectroscopy. Tablet samples were prepared with dried hydrogels dispersed in KBr. The spectra of samples were taken at 400–4000 cm^{-1} wavelength with FTIR-8101 (Shimadzu) for hydrogels prepared in the absence or presence of HA.

Swelling studies

The dried hydrogels were allowed to swell in excess phosphate buffer saline (PBS, pH: 7.4) at 37 °C. The weight of swollen hydrogels were measured for selected time intervals. Triplicate data were obtained for each measurement. Eq. 1 was used to calculate the mass swelling ratio based on dry weight.

$$Q = (W_s - W_d)/W_d \quad (1)$$

where W_s is the weight of swollen hydrogel and W_d is the weight of dried hydrogel.

Determination of density and void fraction

The density of the dried hydrogels was calculated by using Eq. 2.

$$d = W_d/V_d \quad (2)$$

Table 1 Composition of superporous hydrogel composites prepared in this study

Monomer (AAm, 50%)	1,000 μL
Crosslinker (Bis, 2.5%)	200 μL
Water	460 μL
Foam stabilizer (PF 127, 10%)	100 μL
Acrylic acid (AAc)	45 μL
Hydroxyapatite (HA)	250 mg, **1,000 mg**, 2,000 mg
Initiator (APS, 20%)	40 μL
Initiation catalyst (TEMED, 20%)	40 μL
Foaming agent ($NaHCO_3$)	90 mg

where W_d is the weight of dried hydrogel and V_d is the volume of dried hydrogel.

The value of V_d is calculated by solvent displacement method. Hexane was used as the solvent where the hydrogels were submerged in hexane containing cylinder. The hydrogels were quickly removed from hexane and the volume change was determined on the graduated cylinder [2, 16].

The void fraction inside SPHs and SPHCs were determined by immersing the hydrogels in PBS upto equilibrium swelling. The dimensions of the swollen hydrogels were measured and by using these data sample volumes were determined as the dimensional volume. In the meantime, the amount of absorbed PBS into the hydrogels was determined by subtracting the weight of dried hydrogel from the weight of swollen hydrogel. This value is given as the total volume of pores in the hydrogels. The dimensional volume was divided by total volume of pores and this ratio is calculated as the pore volume fraction [17].

Mechanical property studies

The mechanical properties of swollen SPHs and SPHCs were investigated using the Llyod Instruments LR5K (UK) analyzer. The dried hydrogel samples were cut into cylindirical shapes of certain lengths and the initial dimensions were measured in order to calculate the cross-sectional area and the percent of strain. These samples were swollen in PBS upto equilibrium. The swollen dimensions (diameter × thickness) were measured as 2.0 × 1.0 cm for SPH and 1.8 × 1.0 cm for SPHC for compression test. The speed for the test was 5 mm/min and the trigger force was set to 500 N. All measurements were made in triplicate.

SEM/EDAX studies

The morphology and porous structures of SPHs and SPHCs were examined by Scanning Electron Microscopy. Dried hydrogels were coated with a thin layer of palladium gold alloy under vacuum and examined in a SEM (Leo 435 VP, UK). The elements present in the SPHC were shown by Energy Dispersive Analysis by X-ray (EDAX, Oxford, UK).

Cytocompatibility tests

Cytocompatibility studies were carried out with L929 mouse fibroblasts. L929 cell line was obtained from HUKUK Cell Line Collection (No:92123004, Foot and Mouth Disease Institute, Ankara, Turkey). The cells were subcultured in flasks using Dulbecco's modified Eagle's medium (DMEM, Sigma Co., Germany) supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma Co., Germany). The cells were maintained at 37 °C in a humidified CO_2 (5%) atmosphere (Heraus Instruments, Germany).

In order to evaluate the cytotoxicity of SPHs and SPHCs towards fibroblasts, the hydrogels were deposited on confluent cell culture of L929 mouse fibroblasts. The subcultured cells were dissociated with 0.01% trypsin/10 mM EDTA (Sigma Co., Germany), centrifuged and resuspended in medium prior to cell culture in 3.5 cm diameter Petri dishes. When the cells reach confluency ethanol sterilized SPHCs (at equilibrium swelling state) were transferred to the Petri dishes. The morphological alteration of the cells with hydrogel deposit was observed on the 8th hour [18]. The results were compared to a blank constituted of the same cell culture without hydrogel.

Results and discussion

Hydrogel synthesis

In the presented study, acrylamide-based superporous hydrogels and their composites with hydroxyapatite were prepared by solution polymerization technique using ammonium persulphate and *N*,*N*,*N*′,*N*′-tetramethylene diamine as an initiator system. Superporosity was achieved by the means of carbon dioxide gas bubbles generated from the reaction of sodium bicarbonate and acrylic acid. The role of crosslinker, initiaton system, foam stabilizer and foaming agent in the polymerization reaction were discussed in detail in previous studies [2–4, 16]. In our study, the superporous hydrogel composites were prepared with three different amounts of HA. The optimum amount of HA to prepare the hydrogel composites was decided to be 1,000 mg HA per 1,000 μL acrylamide containing mixture, according to experimental results evaluated on swelling kinetics which is described in Section Swelling kinetics, density and void fraction of hydrogels. We observed several advantageous in mechanical properties and swelling behaviour of superporous hydrogels when prepared with HA particles as composite hydrogels. During synthesis, HA particles were distributed inside the SPHC structure homogeneously and no phase separation was observed.

FTIR studies

The FTIR spectra obtained for SPHs and SPHCs are given in Fig. 1. The spectra in Fig. 1c belongs to HA particles. The characteristic peaks for HA at 1,095, 1,045, 965, 610 and 570 cm^{-1} are due to PO_4^{-3} groups corresponding to P–O asymetrical and symetrical streching vibrations and O–P–O bending vibrations [19]. The rotating vibration of hydroxy groups are observed at 635 cm^{-1}. In Fig. 1b for the

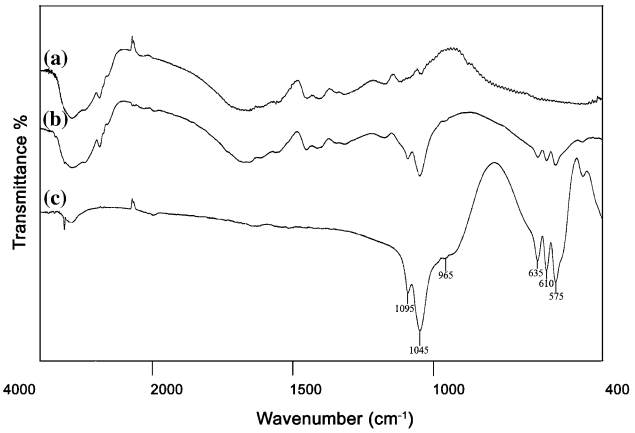

Fig. 1 FTIR spectra of (**a**) SPHs without HA (**b**) SPHCs with HA (**c**) HA particles

composite hydrogel, these characteristic peaks were clearly observed indicating the presence of HA particles in superporous hydrogel structure.

Swelling kinetics, density and void fraction of hydrogels

The swelling profiles for SPHs and SPHCs are shown in Fig. 2. The density, void fraction and swelling ratio for the hydrogels are summarized in Table 2.

As shown in Fig. 2, the extent of swelling for the SPHs was decreased when HA was incorporated into the structure. When 250 mg of HA (per 1,000 μL monomeric mixture) were used, no significant change for swelling values was observed when compared to SPH without HA. In case the HA amount was raised to 1,000 mg, the swelling ratio decreased considerably but the time required to reach the equilibrium swelling for SPHCs was still as fast as SPHs, reaching equilibrium in about 30 s. This result indicates that the interconnected pore structure was

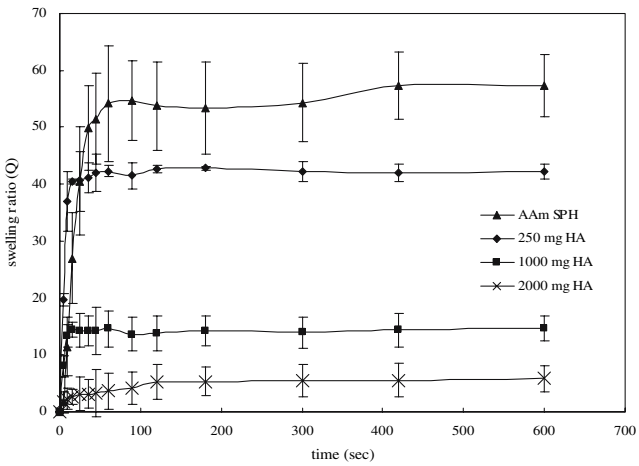

Fig. 2 Swelling kinetics of SPHs and SPHCs. (n = 3, mean ± SD)

Table 2 Density, void fraction and swelling ratio of SPHs and SPHCs

Superporous hydrogels	Density (g/mL)	Void fraction (mL/g)	Swelling ratio
AAm SPH	0.16 ± 0.03	1.13 ± 0.01	54.22 ± 10.17
AAm/HA SPHC	0.35 ± 0.02	0.48 ± 0.03	14.27 ± 2.94

Fig. 3 Photographs of (**a**) SPHC in dried (left) and swollen state (right), (**b**) SPH in dried (left) and swollen state (right)

maintained and the capillary effect to accelerate water absorption was maintained in SPHCs in the presence of 1,000 mg HA. On the other hand, the time to reach equilibrium swelling was raised in the presence of 2,000 mg HA and swelling was hardly observed for the hydrogel composite because of blocking of capillary channels by HA particles. As a result, the optimum amount to prepare the

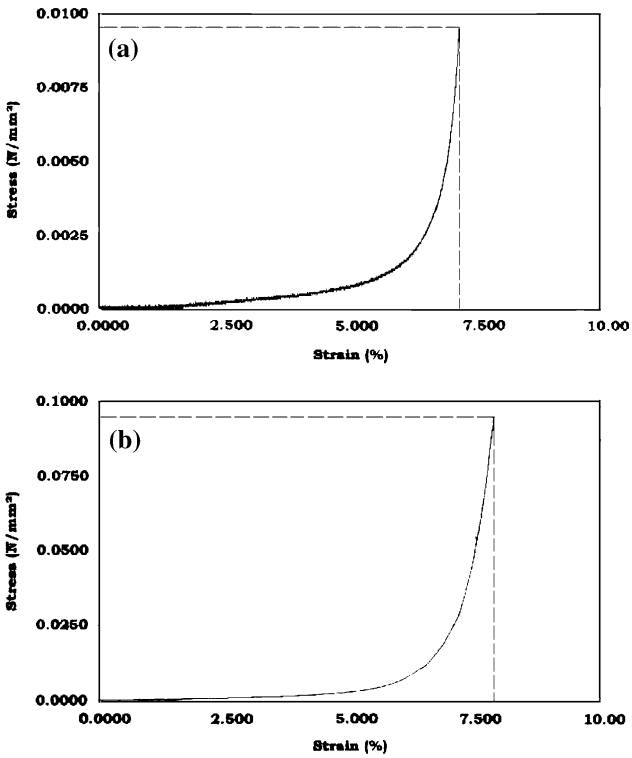

Fig. 4 Compression strength curves of swollen (**a**) SPHs and (**b**) SPHCs

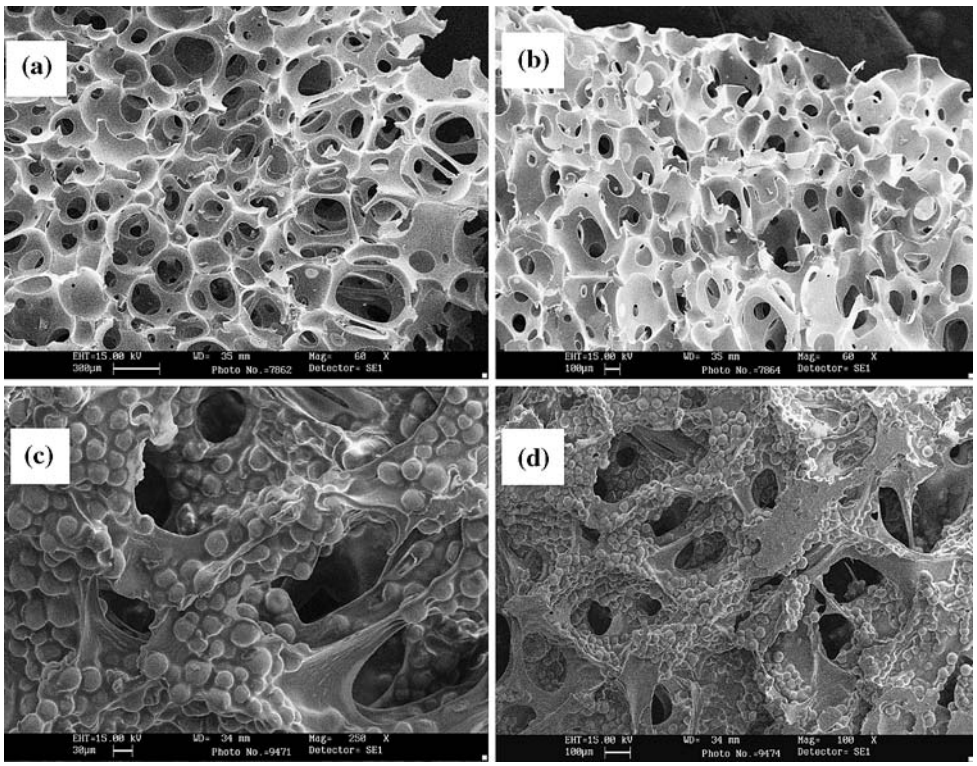

Fig. 5 Scanning electron microscopy photographs of SPHs (**a**) surface ×60, (**b**) cross-section ×60 and SPHCs (**c**) surface ×250, (**d**) cross-section ×100

composite hydrogel was decided to be 1,000 mg of HA per 1,000 μL monomeric mixture. For further analysis and characterization studies described in this sudy, the hydrogel composite with 1,000 mg HA was used.

Figure 3 shows the photographs of hydrogels at dried and swollen states. Swelling causes approximately eight-fold increase in volume of hydrogels. While the swollen SPH is transparent, presence of HA particles in SPHC causes opacity. Besides their applications in controlled delivery systems due to their fast swelling properties [20], the open pore structure allowing the nutrient and waste diffusion is mainly advantageous for these materials to be considered in applications of tissue engineering [1].

As shown in Table 2, the density of SPHs was increased when HA particles were added to the structure due to the increase in the occupied volume. Additionally, the void fraction of the SPHs was decreased with addition of HA particles. These results are consistent with the decrease in swelling ratio. The decrease in void volume leaded a decreased amount of uptake of water into the structure, causing the swelling ratio to decrease in SPHCs.

Mechanical properties

The mechanical properties of hydrogels are important in designing tissue engineering scaffolds as the hydrogel should create and maintain the proper support for tissue development. However, due to their superabsorbent nature, superporous hydrogels are mechanically weak [3, 20]. A recent approach to maintain the necessary stiffness for tissue support, the preparation of superporous hydrogel composites has been suggested. In this study, HA particles were incorporated to the SPHs to synthesize a composite material leading to increased mechanical properties. The

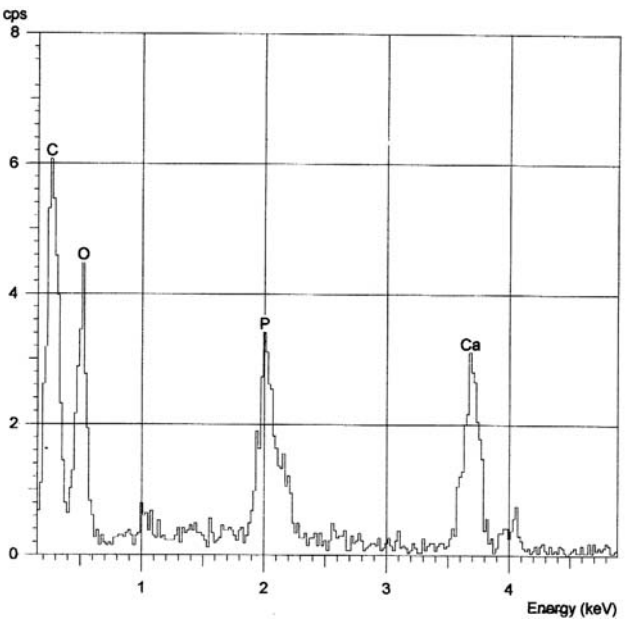

Fig. 6 EDAX spectrum of SPHC

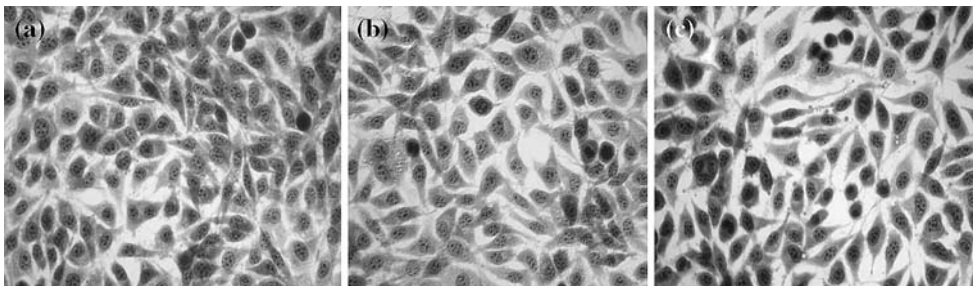

Fig. 7 Light microscopy photographs (×20) of L929 fibroblasts after 8 h of direct contact (**a**) with SPH, (**b**) with SPHC and (**c**) control (TCPS). The cells were stained with Giemsa before imaging

compression strength was improved approximately ten-fold. Based on the slope of compression strength and strain curves shown in Fig. 4, the compressive moduli (E_c) for the SPHs and SPHCs were calculated as 0.63 ± 0.04 and 6.59 ± 0.35 N/mm^2, respectively. Chen et al. also synthesized superporous poly (acrylamide-co-3-sulfopropyl acrylate) hydrogels and added Ac-Di-Sol® powder and observed an improve in the mechanical strength of approximately two-fold [20]. The presence of composite agents in SPHs results in improved mechanical properties and higher modulus polymer networks can be achieved when compared to conventional SPHs.

SEM/EDAX

Figure 5 shows the detailed morphology of SPHs and SPHCs. Higher porosity for SPHs (Fig. 5a and b) was observed compared to SPHCs (Fig. 5c and d) which is in accordance with the results obtained for density and swelling characteristics. The images clearly show that the interconnected pore structure was maintained with the addition of HA particles. The pore size of approximately 100–150 μm remained the same in both hydrogels but the porosity was decreased in SPHCs. The HA particles of size between 25–45 μm were clearly observed in Fig. 5d for the composite hydrogel structure. The EDAX spectrum of the observed area of SPHC showed P and Ca peaks belonging to HA (Fig. 6).

Cytocompatibility studies

The cytotoxicity test was performed by using the direct contact technique, in which the biomaterial to be tested is brought into direct contact with the cells. Figure 7 shows the light microscopy photographs of L929 fibroblasts 8 h after the deposition of hydrogels. No cytotoxicity was observed towards fibroblast cells in vitro. Neither cell death nor morphological disorder was observed throughout the incubation period. It was concluded that SPHs and SPHCs were cytocompatible and do not release cytotoxic compounds in the culture medium in vitro. These preliminary results showed that the SPHCs, with the presence of HA, were suitable for further cell culture studies and shall be useful to provide a suitable microenvironment for 3-D tissue development in vitro in tissue engineering applications.

Conclusion and future perspectives

In the presented study, superporous acrylamide hydrogels and acrylamide/hydroxyapatite hydrogel composites were synthesized and characterized. The addition of HA particles increased the mechanical properties of the superporous hydrogel, keeping the interconnected pore structure. The porous structure is useful to allow the diffusion of metabolites in tissue growth and accomodate the living cells. The HA particles in superporous structure shall act as a reinforcing phase for the matrix, modulating the mechanical properties while permitting the maintanence of biological response. With its structural and biological properties, the synthesized SPHC shall be considered as a novel candidate especially for bone tissue engineering applications. Our future studies are based on osteoblast interaction with this material.

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