ORIGINAL ARTICLE

COLLAGEN-SERICIN-NANO-HYDROXYAPATITE COMPOSITES FOR BONE TISSUE ENGINEERING

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Abstract

The aim of this study was to synthetize porous three-dimensional composites, similar with natural bone structure, for using it in tissue engineering. The new composites prepared by freeze-drying of composite gels consist of a mineral phase - nano-hydroxyapatite and an organic phase - type I fibrillar collagen and silk sericin, the latter acting as a glue-like protein that serves as an adhesive, for binding the collagen fibrils and the mineral phase. The physical-chemical properties were assessed using infrared spectroscopy (FTIR) and thermogravimetry (TGA/DTG); the morphological properties were evaluated by scanning electron microscopy (SEM). The evaluation of the physical-chemical and morphological properties demonstrated that the new composites are promising biomaterials for bone tissue engineering.

Rezumat

Scopul acestui studiu a fost de a sintetiza compozite tri-dimensionale poroase similare structurii osului natural, pentru utilizarea în ingineria tisulară. Noile compozite obținute prin liofilizarea gelurilor compozite conțin o fază minerală - nano-hidroxiapatita și o fază organică – colagen fibrilar tip I și sericină, cea din urmă acționând ca un lipici proteic care servește ca adeziv pentru legarea fibrilelor de colagen cu fază minerală. Proprietățile fizico-chimice au fost determinate prin spectroscopie în infraroșu (FTIR) și termogravimetrie (TGA/DTG); proprietățile morfologice au fost evaluate prin microscopie electronică de baleiaj (SEM). Evaluarea proprietăților fizico-chimice și morfologice demonstrează că noile compozite sunt biomateriale promițătoare pentru ingineria tisulară osoasă.

Keywords: composites, collagen, sericin, nano-hydroxyapatite

Introduction

Since both allograft and autograft have drawbacks, the researchers made continuous attempts to develop bone substitutes, able to provide a strong and biologically compatible 3D micro-porous structure [1]. An ideal scaffold for bone tissue engineering should be a composite material comparable with native bone [2] which should have interconnected porous structure [3, 4], in order to provide a network for cells to attach, to proliferate and to form their extracellular matrix, to allow the flow and circulation of biological fluids and to be resorbed and replaced by newly-formed bone.

It is very well known that bone is composed of the organic, mineral phase, and water. The organic phase in bone is primarily composed of type I collagen [5]. Hydroxyapatite is the main inorganic component of bone and it is recognized as a good bone filler material, because of its osteoconductivity, exceptional biocompatibility, bioactivity in the biomedical application and special structure and function into the body [6]. Thus, composites of hydroxyapatite and collagen have been widely studied during the last decade, being obtained as porous 3D sponges [2, 7-16], collagen fibres with hydroxyapatite particles [17], collagen-hydroxyapatite-hyaluronic acid hybrid composites [18], electro-spinning polyvinyl alcohol-collagen-hydroxyapatite nanofibers [19], collagen-chondroitin sulphate-hydroxyapatite porous composites [20], chitosan-collagen-hydroxyapatite composite matrices [21], nano-hydroxyapatite-collagen-alginate composites [22], collagen-hydroxyapatite-magnetite composites [23].

Based on our previous results [24], about collagen-sericin scaffolds which conclude that these biomaterials could be a suitable 3D culture system for soft tissue reconstruction, due to its good biocompatibility and capacity to support cells differentiation, we extend our study for developing new composites for bone tissue engineering.

In this study, porous three-dimensional (3D) composites were prepared in order to mimic the natural bone structure and to be used in bone tissue engineering. The new composites were synthesized by freeze-drying of composite gels consisting in mineral phase - nano-hydroxyapatite and organic phase - type I fibrillar collagen and silk sericin.

Evaluation of the physico-chemical and morphological properties makes composites proper scaffolds for tissue engineering.

Materials and Methods

Materials

Type I fibrillar collagen in gel form (Coll), with a concentration of 2.54% (w/w), was extracted from calf hide by acid and alkaline treatments as previously described [25]. Sericin silkworm (SS) was purchased from Sigma-Aldrich (Japan). Nanopowder hydroxyapatite (HA) was purchased from Aldrich (USA), the collagenase from *Clostridium histolyticum* was received from Sigma-Aldrich (USA) and GA from Merck (Germany). Sodium hydroxide and phosphate buffer solution (PBS), pH 7.4 were of analytical grade. Chemicals used for cell cultures were supplied by Sigma (Germany) and fetal bovine serum from Gibco. Tissue culture flasks were received from Nunc (Germany).

Preparation of composites

Sericin solution and nano-hydroxyapatite were added to collagen gel and Coll:SS:HA gel composites with the following ratios were obtained: 1:0:0, 1:0.2:0, 1:0.4:0, 1:0:0.3, 1:0.2:0.3, 0:0.4:0.3. Both sericin and collagen concentrations were chosen taking into account our previous results [24]. All the gel composites had the same collagen concentration, 1.2% (reported to dry substance) and they were cross-linked with 0.5% GA, according to the method previously described [26]. After the cross-linking process occurred, the gel composites were lyophilized using the Christ Model Delta 2–24 LSC freeze-dryer (Germany) and 3D porous composites were obtained.

FT-IR analysis

FTIR spectra of composites were registered using a VERTEX 70 BRUCKER FT-IR spectrometer, equipped with an attenuated total reflectance (ATR) accessory. All FTIR measurements were performed in the ATR-FTIR cell on Ge crystal, at room temperature. Spectra were recorded at 4 cm^{-1} resolution, in 4000-600 cm^{-1} wave number region with the obtaining of 32 scans.

Thermal analysis

The thermos gravimetric analysis TGA/DTG curves were registered on a Q500 TA instrument at 10°C/min heating rate, from 25°C to 700°C, under nitrogen atmosphere (balance flow 10 mL/min, oven flow 90 mL).

SEM morphology

The composites morphology, including the internal structure, was assessed by scanning electron microscopy (SEM) analysis of the gold-coated samples. The analysis was performed using a QUANTA INSPECT F SEM device equipped with a field emission gun (FEG), with a resolution of 1.2 nm; gold coating was performed for enhanced surface conductivity.

Results and Discussion

After freeze-drying, the 3D porous composites based on collagen-sericin-hydroxyapatite, with the composition presented in Table I and the aspect presented in Figure 1, were obtained.

Table I

Composition of 3D porous composites

Samples	Coll:SS:HA composition (w/w)
Coll	1.2% (1:0:0)
Coll-SS-20	1 : 0.2 : 0
Coll-SS-40	1 : 0.4 : 0
Coll-HA	1 : 0 : 0.3
Coll-SS-20-HA	1 : 0.2 : 0.3
Coll-SS-40-HA	1 : 0.4 : 0.3

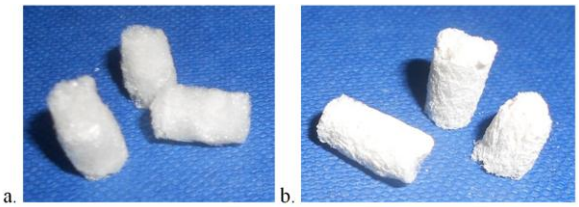

Figure 1.

Example of 3D porous composites: a) Coll-SS-20 and b) Coll-SS-20-HA

The samples from Table I were analysed by FT-IR spectroscopy, thermal analysis (TGA/DTG) and morphological analyses (SEM).

From the FT-IR spectra (Figure 2), the typical bands for collagen can be observed: amide A, B, I, II and III [24].

It is noticed that amide A shifts to lower wave number (from 3313 to 3298 cm^{-1}) probably due to hydrogen bonding. The maximum of Amide II (1549 cm^{-1}) moved to lower wavenumber (1541 cm^{-1}), when sericin was added. This means a destabilization in secondary structure of collagen. Sericin can be conjugated due to the presence of surface-active groups (–OH, –COOH, and $–NH_2$), which can form covalent links [26]. Nevertheless, the triple helix of collagen was not affected by adding sericin and it kept its integrity; the triple helix existence was confirmed by the value of A_{III}/A_{1451} higher than 1 for all studied composites.

Comparing collagen with collagen-hydroxyapatite composite, the characteristic peaks of HA may be observed at around 1061 cm^{-1}, 1034 cm^{-1} and 969 cm^{-1}, which were ascribed to the P–O stretching vibration modes. Thus both sericin and hydroxyapatite influence the structure of composites, especially by hydrogen bonding interactions which lead to more compact structures.

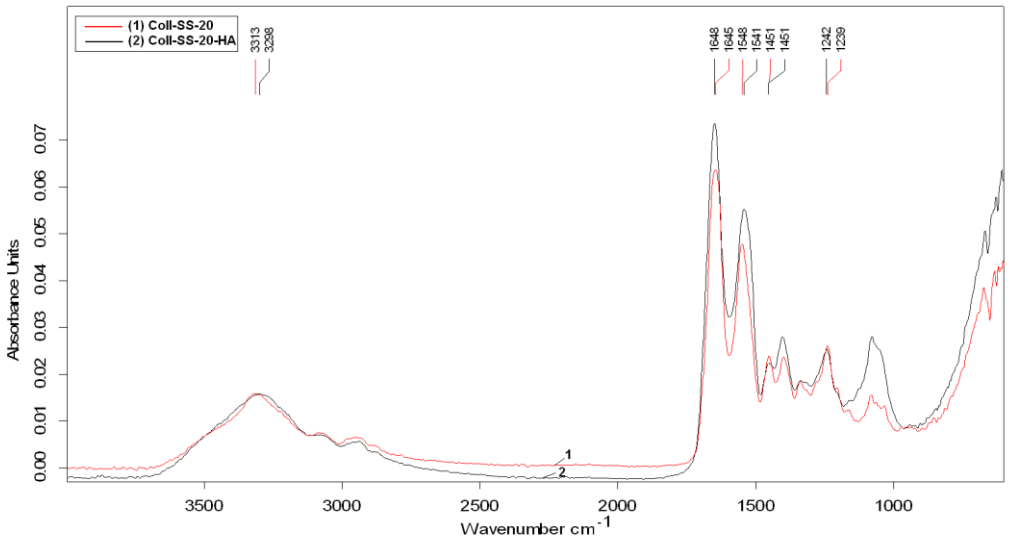

Figure 2.
ATR-FTIR spectra of Coll-SS-20 and Coll-SS-20-HA composites

The hydrothermal stability of protein materials is described by the denaturation temperature, one of the most important parameters in thermal analysis. As Di and Heath [27] showed, during the denaturation process, the collagen fibres undergo hydrothermal contraction which results from the weakening or dislocation of the triple helix conformation at high temperatures, by the breakdown of intermolecular and intramolecular forces (i.e. hydrogen bonding, hydrophobic bonding and crosslink bridges).

As it is shown in Figure 3, collagen reference exhibits a denaturation temperature (T_D) of 48.3ºC, increasing in the samples with higher content of sericin (20 and 40%) to 49.8 and 54.8ºC respectively.

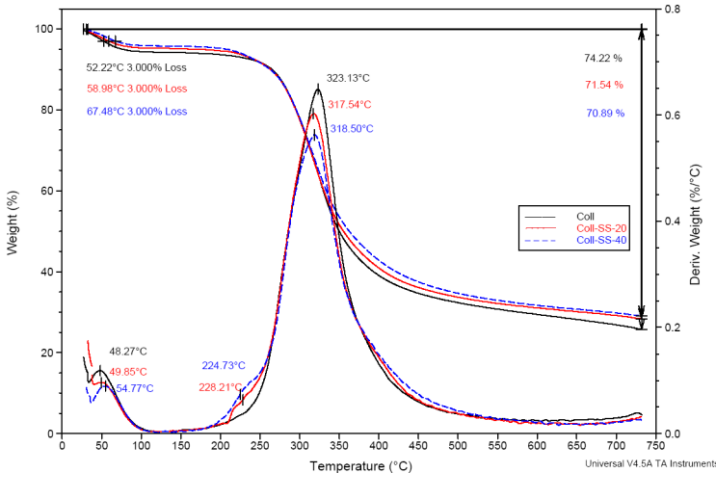

Figure 3.
TGA and DTG curves of Coll, Coll-SS-20 and Coll-SS-40 composites

The temperatures of 52.2, 58.9 and 67.5ºC were registered at 3% weight loss, the samples being more stable if the sericin content is higher due to interactions between collagen and sericin which exhibited the "glue-like" role for collagen fibrils. As Figure 3 and 4 showed, the degradation of the polypeptide chain is initiated between 224÷228ºC, the typical protein polypeptide melting temperature and the maximum degradation rate was found at 315÷323ºC.

In the last stage of thermo-oxidation, the water bound was removed from collagen and side chain groups of amino acid are broken down and peptidic bonds are cleaved. It should be also noted that the residual mass at 700ºC decreases with adding of sericin, from 74.2% in collagen to 71.5% and 70.9% in the composites with 20% and 40% sericin respectively (Figure 3). The same decrease can be noticed for the samples which contain hydroxyapatite. The residual mass decreases from 74.0% in collagen-hydroxyapatite to 64.3% in Coll-HA-SS-40.

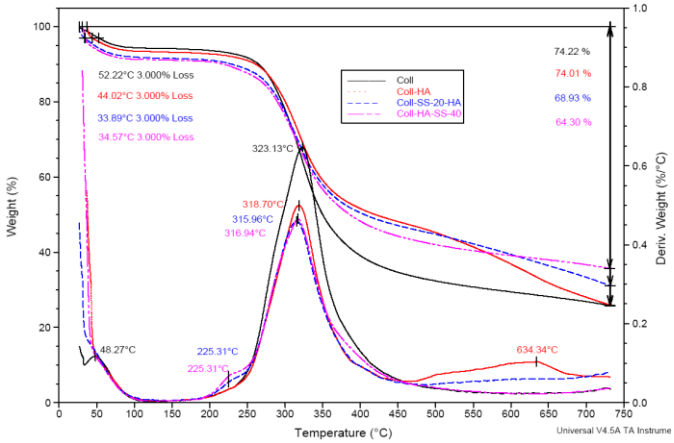

Figure 4.
TGA and DTG curves of Coll, Coll-HA, Coll-SS-20-HA and Coll-SS-40-HA composites

This improvement of thermal degradation resistance of composites including sericin and hydroxyapatite could be due to the interactions into the polypeptide macromolecular network. The porous morphologies were studied by SEM. Figure 5 a-d shows the SEM images for composites. The micro-structure of the composites reveals a porous fibrous collagen network embedded with sericin and nano-hydroxyapatite.

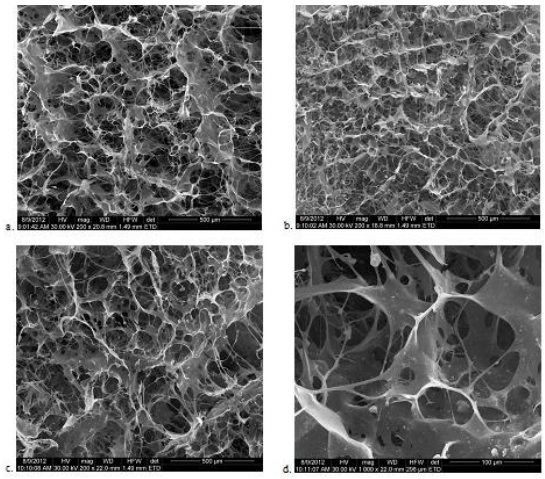

Figure 5.
General morphology of: a) Coll (x200), b) Coll-SS-40 (x 200), c) Coll-HA (x 200) and d) Coll-HA (x 1000) as revealed by SEM

The average of pore sizes measured from the SEM images presented a homogeneus distribution. The pore sizes decreased when sericin was added, the diameter of pores being of 90-150 µm (Figure 5a) for samples without sericin and 50-90 µm (Figure 5b) for sample with highest amount of sericin. As it can be seen from SEM images, comparing Figure 5a with Figure 5c, the pore sizes have very close values for sample with and without nano-hydroxyapatite, varying between 45-115 µm.
This could be explained by nano-hydroxyapatite interaction with collagen and covering the fibrils of collagen with nano particle of hydroxyapatite (Figure 5d). This is observed in detail in Figure 6.

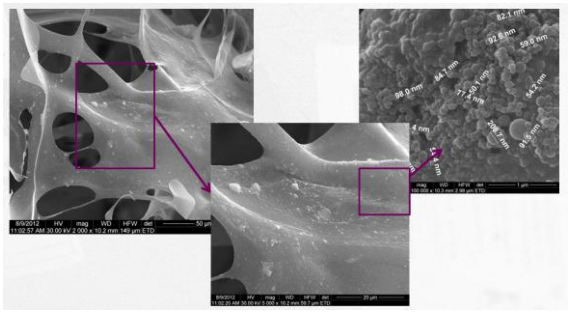

Figure 6.
Detailed morphology (x2000, x5000 and x100000) for Coll-SS-20-HA obtained by SEM

The SEM results are in accordance with FT-IR and TGA/DTG analyses, showing a denser structure with smaller pore sizes if sericin and nano-hydroxyapatite are included in the composite.
The silk sericin in the samples produces dense composites, as SEM analyses showed and reduces water up-take ability. Although the hydroxyapatite did not influence the pore sizes too much, the swelling is lower for composites with hydroxyapatite compared with their reference without hydroxyapatite.

Conclusions

Novel collagen-sericin-nano-hydroxyapatite composites were prepared by lyophilisation. All studied scaffolds kept the integrity of the triple helical structure of collagen. The scaffolds which contain sericin are less thermally and enzymatically stable than the reference sample, but the presence of HA in the scaffold induced a higher thermal stability (as demonstrated by TG/DTG analyses). The scaffolds exhibited a highly interconnected porous structure, having the pore sizes between 45 and 200 µm. The hydrophilicity was the lowest for scaffolds which had the smallest pore size and the denser structure.

Combination of collagen, silk sericin and nano-hydroxyapatite in the ratio of 1:0.2:0.3 and 1:0.4:0.3 (Coll:SS:HA) recommends the new composites as promising biomaterials for bone tissue engineering.

Acknowledgements

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