Fabrication and Characterization of a Porous Composite Scaffold Based on Gelatin and Hydroxyapatite for Bone Tissue Engineering

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Gelatin is a natural protein derived from the organic constituent of bone (collagen type I). Therefore its combination with the natural mineral constituent of bone (HA) is supposed to provide closer properties to the natural bone. In this study, porous scaffolds based on gelatin-hydroxyapatite composite were fabricated by solvent casting method. To increase the biocompatibility of the composite, its fabrication was carried out without using any organic solvent and the porosities of these scaffolds were obtained without using any porogen. The fabrication of porous scaffolds with 6 different compositions (0, 10, 20, 30, 40 and 50 wt% of HA in gelatin, respectively), was followed by characterizing and determining their pore size, morphology, and bending modulus. Cell seeding procedure was carried out using mouse fibroblasts to evaluate the scaffolds' biocompatibility. The scaffolds exhibited pore size range from 50 to 200 micrometers with the good interconnectivity. The obtained bending moduli (above 35 GPa) were higher than those of any other biodegradable scaffolds such as SR-PGA and PLLA/HA that have been reported earlier in the literature. The experiments showed that not only the amount of porosity but also the interconnectivity of pores decreases with increasing HA content. It was also demonstrated that the addition of HA would increase the bending modulus. Cell seeding experiments showed appropriate cell attachments for all the samples.

INTRODUCTION

Bone regeneration has been one of the concerns for human being's health care. Although bone as a dynamic tissue has capability of self-regeneration, but the intensity of the injury restricts it [1, 2]. If the injury is too severe, complete healing will need a substantial approach, which can bring about proper bone regeneration.

Bone tissue engineering is one of such approaches that [3-5] has been practised over the last decades. It is based on the application of
biodegradable materials that can simply eliminate the harmful effects of using metallic implants which may release toxic ions due to corrosion and the subsequent inflammation responses or infection at the implantation site [6-9]. The corrosion of a metallic implant inside the patient’s body also necessitates a second surgery in order to remove the mechanically weakened implant or to prevent additional ion release at the site [8-10]. Another drawback of using metallic implants is attributed to their high modulus in comparison with that of bone. The fact that metals are much stiffer than bone tissue causes stress shielding effect that leads to osteoporosis and thinning of the new bone tissue during healing process, which may increase susceptibility to refractures [8-12].

Using bone graft is another alternative to treat bone defects but this approach can cause some problems as well [13-17]. Allografting may lead to rejection or disease transmission [18], whereas autografting has the disadvantages of the need for second surgery (harvesting) limits supply and the risk of irritation or infection of donor site [16,17].

These problems have made investigators to find a new approach for bone regeneration by using biodegradable materials. This approach, which made a great breakthrough in biomedical science is called Bone Tissue Engineering. Although the techniques of using biodegradable implants in bone tissue engineering are versatile, most of them follow the same pattern: fabrication of a porous scaffold that is based on a suitable biodegradable material, by culturing of osteogenic cells (usually osteoblasts) inside the scaffold and finally the implantation of this mixture into the bone defect [4,5]. After implantation, the biodegradable material starts to degrade and eliminate from the body. Simultaneously the bone cells continue to grow within the pores of vanishing scaffold and eventually a new bone tissue replaces the degrading material.

Polymers, ceramics and composites are three main groups of biodegradable materials used in bone tissue engineering. Synthetic polymers like polylactic acid (PLA), polyglycolic acid (PGA) and their different copolymers (PLGA) have been widely investigated in this field [8,9,18-24]. Naturally derived polymers from organic tissues e.g., collagen [25-31], gelatin [32-35], demineralized bone matrix (DBM), and chitin [36] have been studied as well. Ceramics, especially calcium phosphates such as hydroxyapatite (HA), both in pure form [37-39] and in combination with polymers (composites) [2,10-12,17,40-48] have been demonstrated a great role in bone regeneration. Studies have shown that by using polymers and ceramics together in the form of composites, it can be eliminated some weaknesses and drawbacks of each group, and may be provided a better quality and interface attachment for the new bone tissue [17, 40, 41].

Bone tissue as a composite material constitutes an organic phase (collagen type I) and a mineral phase (hydroxyapatite). Thus, application of composites that are made of these two biomaterials can bring about interesting results. Gelatin, a natural protein derived from the organic phase of bone is much cheaper and more easily obtainable in solutions than collagen. Therefore it has been used as the matrix of tissue engineered porous scaffolds in combination with hydroxyapatite particles as reinforcement. Such scaffolds can be highly effective in bone tissue engineering.

**EXPERIMENTAL**

**Fabrication of Porous Scaffolds**

The procedures of fabrication are approximately the same as described by Yaylaoglu et al. [32]. Details of fabrication procedure used in this paper have been also described [49]. Microbiological gelatin powder specific for cell culture (Merck) was dissolved in distilled water in order to obtain solutions with constant concentrations (12.33 %w/v). Hydroxyapatite (HA) powder (Merck) sieved with mesh No 230 (ASTM) was added to the stirring solution. The mixture was then heated in a water bath to 60°C and was stirred well with a magnet for 1 h to obtain better homogeneity. The uniformed solution was then cast into 6 petri dishes and cooled at room temperature.

Before drying, each membrane was cut into appropriate pieces according to subsequent test standards. Then the pieces were allowed to dry at room temperature for 24 h. Dried pieces were all immersed in glutaraldehyde solution (8% w/v, Merck) for 2 h to be cross-linked. The samples were washed in distilled water for 24 h in order to eliminate any trace of toxic cross-linking solution. The washed water was changed every 6 h.
After this step, all the samples were left to dry at room temperature for 8 h followed by drying at 50°C in an oven for further 24 h. Different HA weight percents, i.e., 0, 10, 20, 30, 40, and 50% were used for samples S0, S1, S2, S3, S4, and S5, respectively (Table 1).

**Scanning Electron Microscopy (SEM)**

Samples with thickness of about 2 mm were cut out for SEM analysis. The samples were sputter coated with gold (approximately 50 nm) under vacuum and then examined by using a Stereoscan 360 model 1992, Cambridge, equipped with an EDXA (Oxford, with Si/Li crystal and EXL program).

**Bending Modulus Measurements with Dynamic Mechanical Thermal Analysis**

Bending modulus of scaffolds was measured by using dynamic mechanical thermal analysis (DMTA) method. Rectangular samples with 40 10 1 mm³ dimensions were prepared and analyzed with a DMTA equipment (Polymer Laboratories, UK). All samples were isothermally tested in 37°C (in dry state). In order to evaluate the viscoelastic behaviour of pure gelatin, for sample S0 the modulus variation with temperature was also examined from -100 to 250°C with a 4°C/min heating rate. All tests were conducted at 1Hz frequency.

**Cell Culture**

In order to evaluate the biocompatibility of scaffolds, mouse L929 fibroblastic cells were used. The cells were maintained in growth medium RPMI-1640 supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin and 10% fetal calf serum. The cell suspension of 4 10⁵ cells/mL was obtained before seeding.

The samples were all sterilized in 70% ethanol for 2 h followed by rinsing for 2 h in sterile distilled water. After sterilization, the samples were then placed in the wells of a polystyrene multiwell culture plate with some wells left empty as negative control. One mL of the cell suspension was poured inside each well and the plate was maintained for 24 h in an incubator with 5% CO₂, 37°C, and 90% humidity. After incubation, the cells were fixed in 50%, 70%, 80%, and 96% ethanol, respectively (the maintenance time in each alcohol solution was 10 min). Since the resolution was enough to detect the cells attachments to the samples and the gelatin's dye or pigment ability, no staining method was used. The samples were directly examined by an optical microscope and the pictures were taken by using Video Capture software.

**RESULTS AND DISCUSSION**

**Scanning Electron Microscopy and X-ray Analysis**

The SEM micrographs of samples showed a porous structure with pore sizes ranging from 50 to 200 microns (Figure 1), which are appropriate for bone tissue engineering applications [50]. The pores had often an oval morphology (due to solvent evaporation) and in many places demonstrated a good interconnectivity with each other. This property is highly important for bone tissue ingrowth inside the scaffold. As it is evident from Figure 1 (a, b, and c), the increase in the amount of HA content has led to a decrease in the number of pores. In pure gelatin (Figure 1a) not only the number of pores was higher but also their morphology was much more oval and interconnected rather than the composite scaffolds. On the other hand, in the composite scaffolds, the average porosity and the degree of observed interconnectivity were highly influenced by the amount of HA. One may conclude that a composite scaffold with a relatively high porosity and good interconnectivity (Figure 1b) may be changed into a structure with more rounded single pores and much less porosity (Figure 1c). This happens when the amount of HA is raised from 20% to 50%.

The most important point is that the porosities obtained in this study for all these samples were achieved spontaneously without using any porogen. Indeed, the natural dissolution of gelatin in water is

<table>
<thead>
<tr>
<th>Sample</th>
<th>HA (wt %)</th>
<th>Gelatin (wt %)</th>
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</thead>
<tbody>
<tr>
<td>S₀</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>S₁</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>S₂</td>
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<td>80</td>
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<td>70</td>
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<tr>
<td>S₄</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>S₅</td>
<td>50</td>
<td>50</td>
</tr>
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</table>
responsible for this porosity, which is attributed to the gelation process of this protein. The temperature of the process (60°C) is well above the gelation temperature of gelatin (35-40°C) [51]. Thus gelatin at such temperature is in its sol state and is constituted of randomly coiled chains distributed in water. During cooling and passing through its gelation point, the polymer solution changes into gel state i.e., the gelatin chains are physically cross-linked together forming a network for entrapment of water molecules [52].

This phenomenon is the consequent of the formation of collagen fold aggregates that like physical bonds cause interlocking of gelatin chains and eventually the gelation process occurs (Figure 2) [51]. After this interlocking and entrapment of solvent molecules inside the gel network, the bulk of gelatin starts to dry during which the trapped water molecules are gradually evaporated and leave some pores instead. Therefore, since the mechanism of porosity formation is based on the dissolution of gelatin, it is evident that the more the composite contains gelatin, it tends to foam further.

Energy dispersive X-ray analysis technique (EDXA) was used to obtain X-ray diffraction spectra from HA particles. The results showed that after fabrication, the calcium to phosphorus molar ratio (Ca/P) of these particles is 1.64, which is close enough to the theoretical molar ratio of HA (1.67).

Dynamic Mechanical Thermal Analysis (DMTA)
In order to evaluate the bending modulus, DMTA method was used. In Figure 3 the differences of storage

![Figure 1. SEM Micrographs from the cross sections of three samples: (a) pure gelatin scaffold exhibits well-interconnected and oval pores, (b) composite scaffold with 20 wt% HA contains less pores but the interconnectivity is maintained, (c) composite scaffold with 50% HA shows single and separated pores with a poor interconnectivity (scale bar represents 200 microns).](image1)

![Figure 2. Formation of collagen-fold aggregates between gelatin chains [51].](image2)
modulus ($E_b$) and tan $\delta$ with temperature for pure gelatin (sample S$_0$) are plotted. The tan $\delta$ curve shows a glass transition temperature ($T_g$) of 221.14°C, which is very close to its theoretical value of 217°C [51]. Apparently the cross-links between gelatin chains are responsible for this rise in $T_g$. Below this temperature the polymer is in glassy state and has a high rigidity but while passing this temperature, molecular vibrations start and consequently a high decrease in bending modulus will occur. Above this point the vibrations are so severe that the chains became more flexible, which causes the polymer to become rubbery [53].

All viscoelastic materials such as polymers and composites have both the viscous and the elastic part, which can be assumed as a dashpot and a spring (joint together), respectively [6, 54]. The elastic part stores energy when a force is applied to the material, while the viscous part always causes loss of energy. Thus the presence of viscous part leads to the development of loss modulus and the presence of elastic part results in the appearance of storage modulus [55]. The relationship between these moduli is given by eqn (1) where $E'$ and $E''$ are storage and loss moduli, respectively and $\delta$ is phase angle:

$$\tan \delta = \frac{E''}{E'}$$  \hspace{1cm} (1)

When the temperature increases, both storage and loss moduli will decrease. However, since the reduction of storage modulus ($E'$) is much more predominant than the loss modulus ($E''$), the $E''/E'$ ratio will undergo an increment. Eventually tan $\delta$ will increase to a maximum value known as $T_g$. Above this temperature the viscous glassy part of the polymer will completely change to the elastic state. An intense decline of tan $\delta$ after this point is predictable, since $E''$ is approaching zero.

For the samples, no significant fluctuations in bending modulus with time were observed during 2 h isothermal analysis. All samples reached an equilibrium state within about 50 min. In Figure 4, the initial and average bending moduli of the samples were compared with each other. As it can be seen from this graph, the increase in HA amount has led to an increase in bending modulus of the scaffold. Although the bending modulus of three samples S$_1$ (10% HA), S$_2$ (20% HA), and S$_3$ (30% HA) were comparable to each other, a much greater rise in bending modulus was observed for S$_4$ (40% HA) and S$_5$ (50% HA) samples. This is due to differences in porosities of the samples. In the latter two samples not only the average number of pores per volume was much smaller but also the pores were much dispersed in comparison with the first ones.

Therefore, it is evident that the mechanical properties such as bending modulus would be obviously higher for HA-rich composites. However, for the first three composites (S$_1$, S$_2$ and S$_3$), since the amount and quality (interconnectivity) of pores are very similar to each other, an increase in HA level has a minor effect on bending modulus. Thus, it can be concluded that the porosity is a much more important parameter affecting the mechanical properties rather than the amount of HA added to the composite system.

The amount of bending modulus obtained in this
Figure 5. Fibroblast cell attachment to the samples: (a) control; (b) pure gelatin (S0); (c) gelatin-10% HA (S1); (d) gelatin-20% HA (S2); (e) gelatin-30% HA (S3); (f) gelatin-40% HA (S4); (g) gelatin-50% HA (S5) (magnification × 250).
work is much higher than those reported earlier for other biodegradable materials used in bone tissue engineering (Table 2) [7, 42, 56].

In Table 2 the bending modulus range of composite scaffolds fabricated in this study is compared with some other biodegradable materials studied earlier in the literature. The comparison is made in dry state for all the samples.

Additionally, the data presented in Table 2 demonstrate the great ability of gelatin/hydroxyapatite scaffolds as the best mechanical support for cell growth in bone tissue engineering applications.

**Cell Culture**

Cell culture experiments showed good cell attachment in all the samples indicating a high level of biocompatibility for the scaffolds. In the 24 h period of cell seeding procedure, a suitable cell response was obtained (Figure 5). For pure gelatin sample (S0), some rounded cells were observed (Figure 5b), which were higher in number than what was for the control sample (Figure 5a).

The evidence of good attachment is the flattened form of the cells, which is observed in all the samples (Figures 5b-5g). A particular substrate is biocompatible if, the fibroblast extend more their body and filopadia on the surface of substrate. For 10% HA composite scaffold (S1), not only the population of attached cells to the surface but also the quality of attachment was better than those of pure gelatin (Figure 5c). More cells have reached webbing and flattening state in Figure 5c rather than in Figure 5b.

Therefore, one can conclude that the addition of 10% HA increases the biocompatibility of the scaffold.

For sample S3 (20% HA), its biocompatibility results was better than S1, since more fibroblast cells were attached suitably to its surface with more extended filopodia and more flattened body (Figure 5d). This demonstrates higher biocompatibility level of this composite in comparison with the previous ones (S0 and S1).

For S3 (30% HA) and S4 (40% HA) samples (Figures 5e and 5f, respectively) although no appreciable increase in cell population was observed, but more cells reached higher stages of attachment (flattening and webbing) rather than S2 sample.

The composite of 50% HA and 50% gelatin showed the best biocompatibility results, which were evident by higher stages of attachments (Figure 5g).

**CONCLUSION**

In this study, a very simple and economical method known as solvent casting was used to fabricate porous composite scaffolds of gelatin and hydroxyapatite, suitable for bone tissue engineering applications.

In order to increase the biocompatibility of the composite, its fabrication was carried out without any organic solvent. The porosity obtained in this study was the natural result of gelatin dissolution in water. This spontaneous porosity will eliminate the necessity of using porogens. The generated pores ranged from 50 to 200 micrometers with a suitable interconnectivity which are appropriate for new bone tissue ingrowth inside the scaffold. Nevertheless, it was observed that not only the amount of porosity but also the interconnectivity of pores decreases with HA content.

DMTA analysis showed a much higher bending modulus for this composite (36-38 GPa) which is quite higher than the amount of dry cortical bone (7-30 GPa) [12,54]. This result is quite promising for bone tissue engineering applications and particular true when compared with the amount reported for other biodegradable tissue engineered materials such as PLLA/HA composites and self reinforced SR-PGA rods. This can demonstrate the high mechanical properties of this scaffold for bone tissue engineering applications. Although increasing the amount of HA level would increase bending modulus, it was observed that

<table>
<thead>
<tr>
<th>Sample</th>
<th>Bending modulus (GPa)</th>
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<tbody>
<tr>
<td>Gelatin + HA</td>
<td>36 - 38a</td>
</tr>
<tr>
<td>PLLA</td>
<td>6.5b</td>
</tr>
<tr>
<td>PLLA + HA</td>
<td>7 - 12.3c</td>
</tr>
<tr>
<td>SR-PGA rods</td>
<td>10 - 15d</td>
</tr>
<tr>
<td>PLDLA + CMP</td>
<td>6.4 - 12.8d</td>
</tr>
<tr>
<td>PLLA + PGA fibers</td>
<td>6d</td>
</tr>
<tr>
<td>Injection molded PGA rods</td>
<td>7d</td>
</tr>
</tbody>
</table>

(a) The present study; (b) [42]; (c) [42,56]; (d) [7].

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**Table 2. Comparison of bending modulus of fabricated scaffolds in this study with some other samples in dry state.**
these differences are dependent much more on the amount of porosity and the morphology of pores rather than HA level itself.

No cytotoxic response was observed during 24 h cell culture in vitro. It was evident that with increasing HA content, a better cell response was obtained indicating a better biocompatibility of HA-rich scaffolds.

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