**In vitro and in vivo study to the biocompatibility and biodegradation of hydroxyapatite/poly(vinyl alcohol)/gelatin composite**

Mingbo Wang,1,2 Yubao Li,1 Jiaqi Wu,3 Fenglan Xu,1 Yi Zuo,1 J.A. Jansen4

1Research Center for Nano-Biomaterial, Analytical and Testing Center, Sichuan University, Chengdu 610064, China
2Chemistry College, Sichuan University, Chengdu 610064, China
3Department of Orthopedics, West China Hospital, Sichuan University, Chengdu 610041, China
4Department of Periodontology and Biomaterials, Radboud University Nijmegen Medical Center, PO Box 9101 6500 HB Nijmegen, The Netherlands

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**Abstract:** A novel porous composite material composed of hydroxyapatite, poly(vinyl alcohol) (PVA), and gelatin (Gel) was fabricated by emulsification. Scanning electron microscopy showed that the material had a well-interconnected porous structure including many macropores (100–500 μm) and micropores (less than 20 μm) on their walls. The composite had a porosity of 78% and showed high water absorption up to 312.7% indicating a good water-swellable behavior that is a characteristic of hydrogel materials. When immersed in water, the scaffold’s weight continuously decreased. After immersion in simulated body fluid, the weight continuously increased because Ca2+ and PO43− ions deposited on the surface and the internal surfaces of the material pores. The deposit was proved to be carbonated hydroxyapatite by thin-film X-ray diffraction, Fourier transform infrared spectroscopy and energy dispersive X-ray analysis. The composite was detected to be non-cytotoxicity by MTT assay. The HA/PVA/Gel material was also implanted subcutaneously in the dorsal region of adult female rats. After 12 weeks of implantation, the porous material adhered tightly with the surrounding tissue, and the ingrowth of fibrous tissue as well as the material’s partial degradation was observed, which partly indicated that the composite was biocompatible *in vivo*. In conclusion, the porous HA/PVA/Gel composite is a promising scaffold for cartilage tissue engineering with more studies.

**Key words:** hydroxyapatite/poly(vinyl alcohol)/gelatin composite; scaffold; SBF; *in vivo*; tissue engineering

**INTRODUCTION**

During physical movement, articular cartilage plays a major role in lubrication as well as reduction of vibration in the joints. As cartilage has a limited potential for self-repair, treatment of cartilage lesions is one of the most challenging problems in orthopedic surgery. Transplantation of isolated chondrocytes has long been acknowledged as a potential method for regenerating the cartilage defects that is damaged or deformed.1 However, recent advance in tissue engineering allow us to aim on the production of larger amounts of cartilaginous tissue, which subse-

Correspondence to: M. Wang; e-mail: wmb20020913@yahoo.com.cn or Y. Li; e-mail: nic7504@scu.edu.cn

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alcohol) (PVA). This material has been selected due to its good biocompatibility and desirable physical properties such as elasticity and water-swelling behavior. Furthermore, hydroxyapatite (HA) has been proven to be bone biocompatible and osteoconductive, and has shown the ability to induce calcification and form a biological bond between implant and subchondral bone.\(^4,5\) Finally, gelatin (Gel) has similar peptide chains as compared with the collagen fibers in cartilage, which may facilitate cellular adhesion and proliferation. The incorporation of Gel can also improve the degradation of a biomaterial.\(^6\)

In view of the earlier mentioned, in the current study a porous HA/PVA/Gel composite was fabricated and evaluated in vitro and in vivo to determine its biocompatibility and biodegradable behavior for its feasibility of cartilage tissue engineering scaffold.

**MATERIALS AND METHODS**

**Materials**

HA slurry prepared by wet method\(^7\) was ultrasounded to diffuse uniformly in deionized water before mixing. PVA (mean degree of polymerization: 1700 \(\pm\) 50) and Gel (AR) were purchased from BeiPei Chemical Agent Factory of Chongqing, China and Tianjin Kermel Chemical Reagents Development Center, China, respectively. Simulated body fluid (SBF) was prepared by dissolving appropriate quantities of the reagents in sterilized water. The reagents were added one by one after each reagent was completely dissolved in 1000 mL water according to the order as listed in Table I. This solution was then adjusted with 1 mol/L HCl and NH\(_2\)C (CH\(_2\)OH)\(_3\) to a pH of 7.4 at 37°C.

**Fabrication of HA/PVA/Gel composite**

HA, PVA, and Gel were added into a three-neck bottle with water, and the weight ratio was 3:6:1. The solution was stirred at 60°C for 2 h and then at 95°C for another 2 h. After the mixture of HA, PVA, and Gel was prepared, emulsifier (Tween 20) was added into a beaker with continuous stirring for 15 min at 800 rpm in order to get a foam mixture with gas bubbles. The foam mixture was then poured into a Petri dish that was exposed to five repeated cycles of freezing at \(-22°C\) and thawing at 20°C. The prepared samples were immersed in water for two weeks that was refreshed every day to dissolve Tween 20. Dried scaffolds were obtained after freeze-drying for 36 h.

**Microstructure of the scaffold**

After sputter coating with gold, the cross-section of dried scaffolds was examined with scanning electron microscopy (SEM) (JSM-5900LV). To evaluate the distribution of HA, five micro-areas (10 nm \(\times\) 10 nm) were randomly selected on the cross-section and the relative contents of Ca and P (atom number) in these micro-areas were detected with energy dispersive X-ray analysis (EDAX, Philip 9100-60). Pore size of the scaffold was measured and the porosity was calculated by the weight and volume of the scaffold before and after immersion in water.

**Swelling test**

Twelve dried samples with a porosity of 78 and 56% were placed at 37°C into culture plates filled with sterile water and SBF, respectively. When swelling equilibrium was reached at 36 h, the samples were taken out and the water on the surfaces was blotted with filter papers. The samples were weighed before and after immersion. Water absorption of the scaffolds in distilled water and in SBF was calculated by a gravimetric procedure.

**Immersion test**

To investigate the bioactivity and degradation behavior of HA/PVA/Gel scaffolds, immersion tests were performed in SBF and distilled water. According to the different immersion fluid and composites’ porosity, immersion test was divided into four groups (high porosity in water, low porosity in water, high porosity in SBF, and low porosity in SBF) and each group had three samples. The samples were immersed in tubes filled with 50 mL SBF or distilled water and placed in a reciprocal shaking incubator shaker at 37°C. Each week, samples were taken out of the soak fluids. Subsequently, these samples were washed with deionized water and dried to constant weight at 60°C. Also, the SBF solution was refreshed every week to maintain the ion concentration that decreased due to deposition of calcium and phosphorus on the specimens. The per-week weight loss of the scaffolds was calculated and plotted until 8 week. The surfaces of the dried scaffolds that had been immersed for 4 and 8 weeks were examined with SEM (JSM-5900LV). After 8 weeks immersion, the deposition on the surface of the samples was characterized with thin-film X-ray diffraction, Fourier transform infrared spectroscopy (FTIR) and EDAX (Philip 9100-60), respectively.

**TABLE I**

<table>
<thead>
<tr>
<th>Order</th>
<th>Reagent</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NaCl</td>
<td>7.996</td>
</tr>
<tr>
<td>2</td>
<td>NaHCO(_3)</td>
<td>0.3500</td>
</tr>
<tr>
<td>3</td>
<td>KCl</td>
<td>0.2440</td>
</tr>
<tr>
<td>4</td>
<td>K(_2)HPO(_4)\cdot3H(_2)O)</td>
<td>0.2280</td>
</tr>
<tr>
<td>5</td>
<td>MgCl(_2) \cdot H(_2)O)</td>
<td>0.3080</td>
</tr>
<tr>
<td>6</td>
<td>1 mol/L HCl</td>
<td>40 mL (volume)</td>
</tr>
<tr>
<td>7</td>
<td>CaCl(_2)</td>
<td>0.2780</td>
</tr>
<tr>
<td>8</td>
<td>Na(_2)SO(_4)</td>
<td>0.0710</td>
</tr>
<tr>
<td>9</td>
<td>NH(_2)C (CH(_2)OH)(_3)</td>
<td>6.057</td>
</tr>
</tbody>
</table>
Evaluation of cytotoxicity

A 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to evaluate the cytotoxicity of the HA/PVA/Gel composite. Because the reduction of the tetrazolium salt into a blue color product (formazan) only occurs in metabolically active cells, the amount of formazan produced is proportional to the number of living cells. By measuring the optical density (OD) value of the formazan, the percentage of viable cells can be determined.

Rabbit fibroblasts provided by the Cell Bank of Peking Union Medical College were used as test cells. MTT agent was prepared by dissolving 5 mg MTT in 1 mL phosphate buffered solution (PBS). The procedure of MTT assay was as follows: (i) Preparation of extraction fluids of the composite: The composite was cut into small pieces (4 mm × 4 mm × 1 mm), which were sterilized by Gamma-ray radiation. The pieces were added to culture flasks, which contained the culture fluid, that is, nutrition medium F12 (GIBCO USA), 10% fetal bovine serum (GIBCO USA), and penicillin–streptomycin solution (100 units of penicillin and 100 μg of streptomycin per milliliter). The culture flasks were placed in an incubator at 37°C and 5% CO2 for 24 h. (ii) Preparation of the cell suspension: Rabbit fibroblasts were grown and passaged in the culture fluid. Third generation fibroblasts were detached with 0.25% papain enzyme and 0.2% ethylenediamine tartrate acid (EDTA). (iii) Replacement of initial cell culture medium: After 24 h of cell culture, for each well of 5 wells in experimental part, the initial cell culture medium was replaced with 0.2 mL extraction fluid, and other 5 wells in experimental part were replaced with 0.1 mL extraction fluid and 0.1 mL earlier-mentioned culture fluid. Each well in the control group was added with 0.2 mL the culture fluid. Finally, the wells plate was put back into the incubator. (iv) Detection of OD value: After 24, 48, and 72 h of incubation, 0.02 mL MTT agent were added to each well. After 4 h of incubation, 0.15 mL dimethyl sulphoxide (DMSO) was added to each well and the culture plate was slightly shaken for 10 min. The OD value of each well was assessed with an enzyme-connection immunodetection apparatus (BIO-RAD Model 550).

Cell shape

Rabbit fibroblasts were grown in the culture medium composed of the nutrition medium F12, 10% fetal bovine serum and penicillin-streptomycin solution (100 units of penicillin and 100 μg of streptomycin per milliliter). Third generation fibroblasts were suspended at a concentration of 9.5 × 104 cells/mL after detachment with 0.25% papain enzyme and 0.2% ethylenediamine tartrate acid (EDTA). Composite material was cut into pieces (18 mm × 18 mm × 1 mm) and sterilized by Gamma-ray radiation. The square-shaped composite discs were placed at the bottom of each well of a 12 wells plate and 1 mL of cell suspension was added to each well. In one well of each 12 well plate, no composite material was placed. This well was used as control surface. Subsequently, the 12-well plates were incubated at 37°C and 5% CO2. After 24 h of incubation, the supernatant liquid was removed and each well was supplemented with 1 mL culture fluid and 0.005 mL adenovirus-epidermal growth factor receptor (AD-EGFR) that was used as fluorescein agents. Subsequently, the plates were put back into the incubator for another 72 h. Then, the shape of the fibroblasts in the experimental group and control group was examined with an inverted microscope (Olympus) under the background of fluorescence.

RESULTS AND DISCUSSION

Microstructure of the scaffolds

SEM micrographs of the cross-section of HA/PVA/Gel scaffold (Fig. 1) revealed two distinct pore sizes: (i) macro pores of 100–500 μm, and (ii) micro pores of less than 20 μm, which were located on the walls of the macro pores. All pores formed a well-interconnected network. It is obvious that the porous structure is beneficial for tissue ingrowth and flow transport of nutrients and metabolic waste. From the micrographs, the evident aggregates of HA cannot be observed. In Figure 2, the approximate contents of Ca and P value in five micro-areas of the cross-section of the scaffold indicate that the HA particles are uniformly distributed in the polymer matrix. It is important for the final mechanical and biological performance that inorganic particles uniformly distribute in particle-reinforced composites.
By measuring the weight and volume of the composites before and after soaking in water, the porosity \( P \) of the composite was calculated via the following equation [Eq. (1)]:

\[
P = \left( 1 - \frac{M_d \times V_s}{M_s \times V_d} \right) \times 100%
\]  

(1)

where \( M_d \) and \( V_d \) are the weight and volume of dried scaffold, and \( M_s, V_s \) are the weight and volume of swollen scaffold. Using this formula, the porosity of the scaffold, as shown in Figure 1, was between 50 and 80%. By changing the parameters, scaffolds with different porosities can be fabricated according to specific requirements.10

**Swelling test**

A gravimetric procedure11 was carried out to monitor the water absorption of two dried HA/PVA/Gel composites with porosity of 78 and 56%. Water absorption \( A \) was calculated via the following equation [Eq. (2)]:

\[
A = \frac{W_s - W_d}{W_d} \times 100%
\]

(2)

where \( W_s \) and \( W_d \) are the weight of swollen and dried gel. The results (Table II) show that the HA/PVA/Gel composite with 78% porosity has water absorption of about 300% due to excellent hydrophilic nature of PVA and Gel. The maximum water absorption is 312.7% in water and 294.3% in SBF. Highly porous scaffolds possess a high water absorption, which is attributed to the presence of an increased space to hold endosmic water as well as an increased contact area to react with water. This indicates that the water content can be partly controlled by changing the composite porosity. Hydrogel is used for the manufacture of the replacement of soft tissue, such as cartilage, intraocular tissue, and so forth, just because of its excellent property of being water-swellable and the consequent lubricative nature, which plays a key role in the function of vibration reduction.12

The data as listed in Table II also show that the water absorption in water is higher than in SBF, which is explained by the fact that with the addition of the ions in SBF, the osmotic pressure of water decreases between the exterior and interior of the swelling gels. This phenomenon has been reported before by other researchers.13

**TABLE II**

<table>
<thead>
<tr>
<th>Immersion Fluid</th>
<th>Water (%)</th>
<th>SBF (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porosity (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td>78</td>
<td>304.4</td>
</tr>
<tr>
<td>Group 2</td>
<td>56</td>
<td>305.3</td>
</tr>
<tr>
<td>Group 3</td>
<td>78</td>
<td>312.7</td>
</tr>
<tr>
<td>Average</td>
<td>56</td>
<td>307.5</td>
</tr>
</tbody>
</table>

Figure 1. SEM micrographs of HA/PVA/Gel composite: (A) a cross-section of the scaffold, (B) micropores on the walls of the macropores.

Figure 2. The distribution of the relative contents of Ca and P in the composite. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
Immersion test

Weight loss analysis

High porosity (78%) and low porosity composites (56%) were immersed in water and SBF solution. They were weighed before and after immersion and the weight loss per week was calculated according to the following equation [Eq. (3)]:

\[
\text{Weight loss} = \frac{W_i - W_d}{W_i} \times 100\% \tag{3}
\]

where \(W_i\) and \(W_d\) are the initial dried weight and post-immersion dried weight of the composites.

Figure 3 shows the weight loss of composites with a different porosity in water and SBF at various time intervals. Each point in Figure 3 combines the average value of three samples and their standard deviations. It can be seen that: (i) the weight loss in water continually increases during time for high porosity and low porosity composites, and that the high porosity composite shows a relative high weight loss within the same time interval. The weight loss is mainly attributed to the degradation of Gel. (ii) In SBF, the weight loss of high and low porosity composites continuously decreases from 1 to 8 weeks so that at the end of 8 week the sample’s weight far surpasses the weight before immersion. This is attributed to the degradation of the composites as well as the deposition of Ca\(^{2+}\) and PO\(_4^{3-}\) ions out of SBF on the composite and pore surface. The weight loss decreases when the deposition dominates. (iii) In SBF, because of a better permeability of the ions and the higher surface area of the composites, the deposition of Ca\(^{2+}\) and PO\(_4^{3-}\) ions is faster in high porosity composites than in the low ones. Therefore, the decrease of weight loss of the high porosity composites is also faster.

Hydrolysis of peptide bonds and cross-links is the main mechanism of Gel degradation in a non-enzymatic solution. Thus, degradation of the scaffold is attributed to the hydrolysis of Gel. In this test, the hydrolysis of the composites can be promoted by persistent shaking at 37°C simulating the in vivo conditions. The presence of cross-linking via chemical bonds in HA/PVA/Gel composite has been verified in another publication. The prolonged degradation time confirms that HA/PVA/Gel composite has a good degree of cross-linking, because pure Gel completely takes a degradation within a conspicuous short time.

Characterization of the mineral deposit

SEM micrographs of the scaffolds immersed in SBF for 4 and 8 weeks are presented in Figure 4. They show that some minerals deposited on the pore walls of the composites after 4 weeks of immers-
sion [Fig. 4(A)]. After 8 weeks of immersion [Fig. 4(B)], a lot of minerals covered the surface and the internal walls of the pores. The thin-film X-ray diffraction of the mineralized deposit is depicted in Figure 5. The characteristic peaks of HA locating at 25.9°, 31.8°, 40.0°, 46.7°, 49.5° appear clearly in the spectrum of the deposit. The FTIR pattern of the mineralized deposit (Fig. 6) presents the characteristic peaks of PO$_4^{3-}$ at 1032 and 1087 cm$^{-1}$ and the peak at 1320 cm$^{-1}$ corresponding to hydroxyl bending vibration. Moreover, the peak at 1423 cm$^{-1}$ indicates the presence of CO$_3^{2-}$ in the deposit. EDAX analysis confirms that Ca/P ratio value of the deposit is 1.75 that is beyond 1.67 of HA due to the presence of CO$_3^{2-}$. These results manifest that the deposit is a carbonated HA that is similar in composition to bone apatite. The deposition phenomenon proves that the composite is a bioactive material, which can induce a biological bond with bone.

Evaluation of cytotoxicity

An MTT assay with rabbit fibroblast was done to evaluate the cytotoxicity of the composites. The relative growth rate (RGR) of the fibroblast in the extraction fluid of HA/PVA/Gel composite was assessed. RGR was calculated via the following equation [Eq. (4)]:

$$\text{RGR} = \frac{\text{OD}_e}{\text{OD}_c} \times 100\% \quad (4)$$

where OD$_e$ and OD$_c$ are the average OD value of the experimental and control groups. The results (Table III) show that RGR decreases with culture time and increases with a decreasing concentration of extraction fluids, which corroborates with other studies. The data also demonstrate that the fibroblasts have a high RGR in the composite extraction fluid, which proves a good cell-viability. The toxicity grade (CTG) of the composite is obtained by the relationship between RGR and CTG according to standard GB/T 16886-1997 (Table IV). CTG of the composite is grade 1 at 24 and 48 h, indicating non-toxicity. At 72 h, the CTG of the composite increases to grade 2, but it is concluded that the composite is non-toxicity by referring to the intensive viability of the fibroblasts at 72 h of cell culture in Figure 7.

Cell shape

Figure 7 show that the fibroblasts are a long fusiform shape with copious endochylema and an elliptical nucleus. The fibroblasts proliferate on the experimental composite surfaces and some cells revealed cleavage, which was confirmed by the observation of separating nuclei. The viability of the cells was on a high level, as indicated by the presence of a great number of living cells. This proves that the HA/PVA/Gel composite is cell compatible.

| TABLE III | RGR and CTG of Rabbit Fibroblasts Grown in the Extraction Fluid of HA/PVA/Gel Composite |
|-----------|---------------------------------|-----------------|-----------------|-----------------|
|           | RGR (%)                         | 24 h            | 48 h            | 72 h            |
|           | CTG                             | 24 h            | 48 h            | 72 h            |
| Concentration of Extraction Fluid (%) |                   |                 |                 |                 |
| 100       | 83.6                            | 75.4            | 54.0            | 1               | 1               | 2               |
| 50        | 83.7                            | 81.0            | 55.6            | 1               | 1               | 2               |

Figure 5. Thin-film X-ray diffraction pattern of the mineralized deposit.

Figure 6. FTIR spectrum of the mineralized deposit.
In vivo evaluation

Evaluation of the retrieved composite implants was done by macroscopic observation and light microscopy. After 1 week of implantation, the implants were located in a subcutaneous pocket that was clearly separated from the surrounding tissue. At 4 weeks, fibrous tissue formation started resulting in encapsulation of the implant. At 12 weeks, the implants adhered tightly to the surrounding tissue, and the district of the implantation resembled the surrounding original tissue.

Light micrographs of histological sections after 1, 4, and 12 week(s) of implantation are depicted in Figure 8. At 1 week [Fig. 8(A,B)], a lot of inflammatory cells were seen within and around the implant. Evidently, a phagocytic and inflammatory reaction occurred. After 4 weeks of implantation [Fig. 8(C,D)], the number of inflammatory cells decreased sharply, but the fibrous capsule was still present. Fibrous tissue was growing into the implant pores. At 12 weeks, the implant was completely penetrated with fibrous tissue. Further, it was noticed that the surrounding fibrous capsule became much thinner [Fig. 8(E)]. The implant had partially degraded, which could be associated with a very mild inflammatory response [Fig. 8(F)]. As a lot of enzymes are present in vivo, the partial degradation of the HA/PVA/Gel composite is mainly attributed to enzymolysis of Gel.\textsuperscript{16,22}

CONCLUSION

A novel HA/PVA/Gel composite was prepared by an emulsification approach. The porous microstructure of the HA/PVA/Gel composite was found to be well-interconnected via a lot of micropores on the walls of macropores. The pores with a size of 100–500 \( \mu \)m in diameter allowed the ingrowth of tissue. The porous composite shows excellent water-swelling behavior and high water absorption up to 294.3\% in SBF. After 8 weeks of immersion, the weight loss of the composite increased in water and decreased in SBF. At the end of 8-week immersion, a lot of apatite crystals that had proved to be carbonated HA were formed on the surface and internal walls of the pores. The composite was also confirmed to be nontoxic and cell-compatible by MTT assay and cell culturing.

In subcutaneous implantation in rats, an inflammatory reaction was seen during the initial stage of implantation. After 12 weeks of implantation, fibrous tissue had grown into the porous composite implants. The occurrence of local defects in the composite indicates that the material is biodegradable.

On the basis of earlier mentioned text, we conclude that the in vitro and in vivo results show that the HA/PVA/Gel composite posses good characteristics so that it can be further researched as a scaffold material for cartilage tissue engineering.

<table>
<thead>
<tr>
<th>CTG</th>
<th>RGR (%)</th>
<th>Toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>≥100</td>
<td>No</td>
</tr>
<tr>
<td>1</td>
<td>75–99</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>50–74</td>
<td>Slightly*</td>
</tr>
<tr>
<td>3</td>
<td>25–49</td>
<td>Midrange</td>
</tr>
<tr>
<td>4</td>
<td>1–24</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>Obvious</td>
</tr>
</tbody>
</table>

\*Evaluated by referring to the viability of cell cultured.

Figure 7. Micrographs of the fibroblasts under background of fluorescence after 72 h of culture. Experimental group (A) was cultured on the HA/PVA/Gel composite and the control group (B) was the bottom of a well dish. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
Figure 8. Light micrographs of HA/PVA/Gel composite, which was subcutaneously implanted in the dorsal region of adult female rats for 1 (A, B), 4 (C, D), and 12 weeks (E, F). The regions as indicated by black squares in (A, C, and E) were magnified to (B, D, and F). Black arrows in (B, D, and F) show ingrowth of tissue and white arrows in (B, D, and F) show the presence of inflammatory cells. All sections were stained with H&E. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
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References