Gelatine-Hydroxyapatite Nanocomposites for Orthopaedic Applications

U. Vidyarthi, P. Zhdan, C. Gravanis and C. Lekakou

Abstract—**This study focuses on the preparation and testing of hydroxyapatite-gelatine nanocomposite gels via a sol-gel route and in situ formation of hydroxyapatite (HA) type salts. Four types of gels and foams were prepared with Ca/P molar ratio of 0.43 and 0.86 and hydroxyapatite/gelatine weight ratio of 0.50 and 0.70. Crosslinking of gelatine chains was carried out in 1% glutaraldehyde and dynamic mechanical torsion tests were used to measure the viscoelastic properties of the gels and foams, optimize the crosslinking time and assess their mechanical performance. Optical and atomic force microscopy were used to investigate the micro- and nano-structure of the produced composites: in these studies, it was confirmed that the gels were nanocomposites with a nano-structure very similar to that of bone and several similarities in the microstructural features. The best foams incorporated dual pore size distribution.**

***Index Terms*—Gelatine, hydroxyapatite, nanocomposites, orthopaedic**

I. Introduction

The focus of this study is to develop materials for tissue engineering in orthopaedic applications, i.e. to replace cartilage and bone. The traditional approach of research in this area is to develop a three dimensional porous scaffold, permeable to cells such as osteoblasts, for cell culture permeation, attachment and proliferation. This raises the opportunity for the scaffold to be implanted and for the cells to proliferate and generate extracellular matrix in vivo, while the scaffold will slowly degrade. However, the growth of very small amounts of bone in vivo in this manner may take several weeks and months [1-4]. The proposal in this study is to create a biomimetic material that closely resembles bone and could incorporate the appropriate cells.

Manuscript received March 5, 2007

U.Vidyarthi: Centre for Biomedical Engineering, University of Surrey, Guildford, Surrey GU2 7XH, UK

P.Zhdan, C.Gravanis and C.Lekakou, School of Engineering, University of Surrey, Guildford, Surrey GU2 7XH, UK

Bone is a complex composite with a hierarchical structure. At the macrostructural level (>500 μm) there are differences between the cortical and cancellous bone [5-7]: Cortical bone, which comprises the main part of long bones, is dense with only 5-10% porosity and consists of cylindrical osteons with their axis parallel to the axis of the bone, where each osteon consists of wrapped lamellae of 3-7 μm thickness each, with each lamella being a bed of aligned collagen fibres; adjacent lamimae might have different fibre orientations, from longitudinal to transverse. Cancellous bone at the end of long bones is highly porous (spongy) with 50-90% porosity. Both types of bone have collagen microfibrils, which contain lamellae of self-assembled, aligned collagen nanofibrils [5], comprising triple helix collagen type I molecules of about 300 nm length and 1.5 nm diameter [8]. Type I collagen molecules form D-periodic cross-striated fibrils with an axial periodicity D = 67 nm, providing the biomechanical scaffold for cell attachment and anchorage of other macromolecules [8, 9]. In small gaps between these lamellae of collagen nanofibrils are embedded hydroxyapatite (HA) crystals of about 50x25x2 nm [10-12]. Cortical bone contains about 60% HA, 16% collagen, 23% water and 2% ground substance [5]. Cartilage consists of a collagen gel with some elastic fibres and a matrix of proteoglycans (sulphates). Cartilage has viscoelastic properties with a Young's modulus in the range of 0.3 to 1.5 MPa [13] whereas cortical bone has a Young's modulus of 17 GPa in the axial direction [14].

HA particles have been deposited [15] on the surface of collagen or gelatine fibres or gels or incorporated [16-19] in the microstructure of collagen or gelatine gels. We have considered that the latter case is preferable as there is a chance to mimic bone structure in this manner. It has been found that [20] nanocomposite gelatine-HA scaffolds are better for the attachment and proliferation of osteoblasts than microcomposites. In many of these studies as in the present study, gelatine has been used as a low cost source of soluble collagen.

II. Materials and Experimental Techniques

Powder of animal gelatine was used as a low cost source of soluble, denatured collagen, consisting of amino-acid chains. The preparation technique of gelatine/hydroxyapatite gels in this study consists of dissolving the gelatine in water and produce hydroxyapatite from an in situ reaction in the gelatine

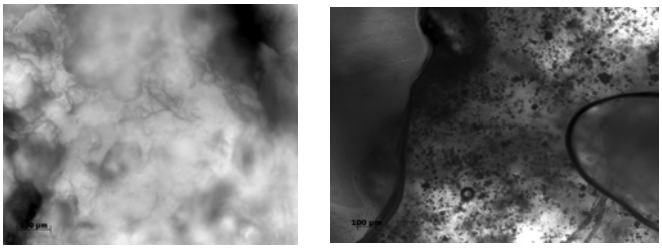
solution, so that hydroxyapatite is still dissolved in this solution; then both gelatine and hydroxyapatite are precipitated in the form of a colloidal suspension, which will eventually generate the nanocomposite gel.

The in situ reaction involved the starting materials of $Ca(OH)_2$ and H_3PO_4; their reaction may produce different types of salts, such as $Ca_3(PO_4)_2$, if the Ca/P molar ratio is higher than 1.67, or $CAHPO_4$ otherwise, where the former has been reported to lead to the production of mechanically weak scaffolds [21]. Molar ratios Ca/P of 0.43 and 0.86 were investigated in this study. In all cases the amount of gelatine was calculated to produce nanocomposites of two weight ratios of "hydroxyapatite"/dry gelatine, 0.50 and 0.70. The produced gels were left in 1% glutaraldeyde to effect crosslinking of the denatured collagen chains. Different crosslinking times were tried, 5.5, 7 and 8 h, to optimize the process.

The gelatine powder was dissolved in 9.55 ml of 1M H_3PO_4 under stirring. 0.1 M $Ca(OH)_2$ solution was added under stirring and the solution was filled to 160 ml with distilled water. Then, the pH was changed to 9 by addition of 1M NaOH, in which case a colloidal whitish suspension of gelatine and "hydroxyapatite" was formed. The colloidal suspension was left to rest for 18 h for the gelatine and "hydroxyapatite" particles to precipitate. After filtering, the precipitates were placed on aluminium discs and stored in the fridge for 15 h for the gels to form. Then, they were placed in 1% glutaraldeyde in the fridge for 5.5, 7 or 8 h to optimize the crosslinking time.

All specimens were subjected to DMA torsion tests to evaluate their mechanical performance; the best specimens were examined under AFM to study their structure hierarchy and the HA particles. In general the produced gels of HA and gelatine were nanocomposites of HA dispersed in the gelatine matrix. Fig.1(a) displays an optical micrograph of such a gel which, although it shows a microfibrillar structure regarding the gelatine matrix, shows no HA microparticles. If, however, the pH of the gelatine solution in 1M H_3PO_4 was changed to 9 by adding NaOH and then the required amount of 0.1 M $Ca(OH)_2$ was added under stirring, precipitation of gelatine particles occurred first, followed by formation and precipitation of "HA" microparticles, which resulted at the end in a microcomposite gel, as is evident in the optical micrograph of Fig.1(b) that clearly shows dispersed "HA" microparticles in the gelatine matrix. It was easily noticed that the "HA"/gelatine microcomposite gel was fragile and brittle, easily fracturing into small fragments when handled. The nanocomposite samples, however, were much stronger and remained intact when handled, especially those with the optimum crosslinking time.

In order to produce foams, gel samples were first frozen in a freezer at -25 ^oC for 12 h and then freeze-dried in a Heto LyoLab 3000 for 4 days. In this manner, they exhibited a water loss in the range of 92 to 97 %. The so produced foam samples were subjected to DMA shear tests and examined under the optical microscope.



(a) (b)

Fig.1. Optical micrographs of "HA"/gelatine gels
(a) nanocomposite (b) microcomposite

III. DMA RESULTS: OPTIMISATION OF CROSSLINK TIME

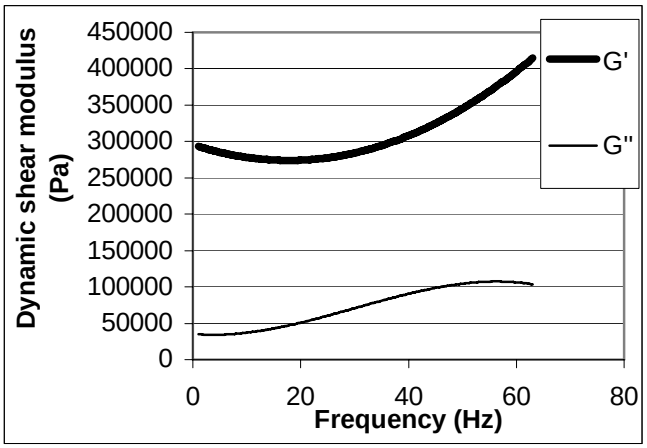
DMA was carried out in an RDA II Rheometrics with the specimens in dynamic torsional mode at two alternative strains, 0.1 and 1%, subjected to a frequency sweep from 1 to 60 Hz at a constant temperature of 37 ^oC. The results included viscolastic properties such as elastic shear modulus, G', loss shear modulus, G", and tanδ. The optimization of the collagen crosslinking time was performed on the basis of tested samples with a Ca/P molar ratio of 0.43, which was expected to be the best Ca/P ratio on the basis of past studies [21]. The elastic modulus G' generally increased with increasing frequency in DMA, as expected due to the viscoelastic nature of the gels.

Table 1. DMA results of maximum elastic shear modulus, G' in MPa, during a frequency sweep from 1 to 79 Hz, at 37 ^oC, after different crosslinking times of gels in 1% glutaraldehyde. (Ca/P molar ratio = 0.43).

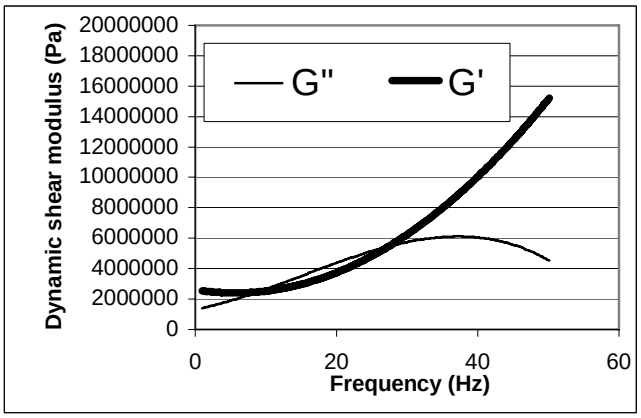
HA/ Gelatine	Crosslinking time: 5.5 h		Crosslinking time: 7 h		Crosslinking time: 8 h	
	Strain 0.1%	Strain1 %	Strain 0.1%	Strain 1%	Strain 0.1%	Strain 1%
0.50	0.15	0.06	0.40	0.06	0.08	0.08
0.70	0.03	0.008	17.6	4.70	0.09	0.12

Table 1 presents the DMA results for the maximum G' value during the frequency sweep for the various tested samples. It can be seen that, in general, 7 h crosslinking is the optimum time yielding the highest value for G'. Fig.2(a) and (b) present the graph of the measured viscoelastic properties for the nanocomposite gels with "HA"/dry gelatine weight ratio of 0.50 and 0.70, respectively, as a function of frequency at a strain of 0.1% and a constant temperature of 37 ^oC, after they were crosslinked in 1% glutaraldeyde for the optimum time of 7 h. Both gels seem viscoelastic with the same order of G' and

G". The nanocomposite gel with "HA"/dry gelatine=0.70 had a higher elastic modulus than the nanocomposite gel with "HA"/dry gelatine = 0.50, and both gels remained intact after being handled and tested.



(a)



(b)

Fig.2. DMA graphs of "HA"/gelatine nanocomposite gels at 0.1% strain and 37 ^{o}C, after 7 h of crosslinking in 1% glutaraldeyde. "HA"/dry gelatine = (a) 0.50 and (b) 0.70 g/g

IV. DMA RESULTS: "HA"/GELATINE NANOCOMPOSITE GELS WITH DIFFERENT Ca/P RATIOS

Table 2 presents the results of the maximum elastic shear modulus, G', from the DMA torsion tests for the different "HA"/gelatine nanocomposite gels after 7 h of crosslinking. In general, very high calcium content (Ca/P = 0.86 and "HA"/gelatine = 0.70 g/g) led to a brittle gel which also had the lowest value for the maximum G'. The best gel was that with a molar ratio Ca/P = 0.43 and a weight ratio of "HA"/gelatine = 0.70 g/g: this gel demonstrated the highest value of maximum G' (17.6 MPa) while it still displayed viscoelastic behaviour as is shown in Fig.2(b). From the foam samples (see Table 3), the nanocomposite foam with Ca/P = 0.86 and "HA"/gelatine = 0.50 g/g exhibited the highest elastic shear modulus. The sample was highly elastic with a low loss modulus.

Table 2. DMA testing of gels: maximum elastic shear modulus, G' in MPa, during a frequency sweep from 1 to 79 Hz, at 0.1% strain and 37 ^{o}C, after 7 h in 1% glutaraldehyde.

"Hydroxyapatite"/ Dry gelatine (g/g)	**Ca/P molar ratio**	**Maximum G' (MPa)**
0.70	0.86	0.025
0.70	0.43	17.6
0.50	0.86	0.700
0.50	0.43	0.400

Table 3. DMA testing of foams: maximum elastic shear modulus, G' in MPa, during a frequency sweep from 1 to 79 Hz, at 0.1% strain and 37 ^{o}C, after 7 h in 1% glutaraldehyde.

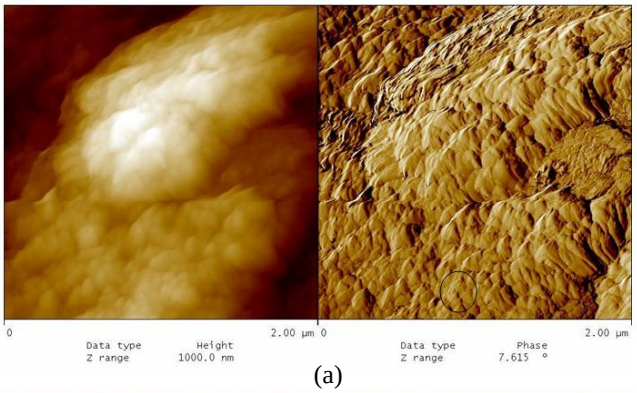
"Hydroxyapatite"/ Dry gelatine (g/g)	**Ca/P molar ratio**	**Maximum G' (MPa)**
0.70	0.86	0.01
0.70	0.43	fragile
0.50	0.86	1.10
0.50	0.43	0.03

V. MICRO- AND NANO-STRUCTURE OF "HA"/GELATINE GELS AND FOAMS

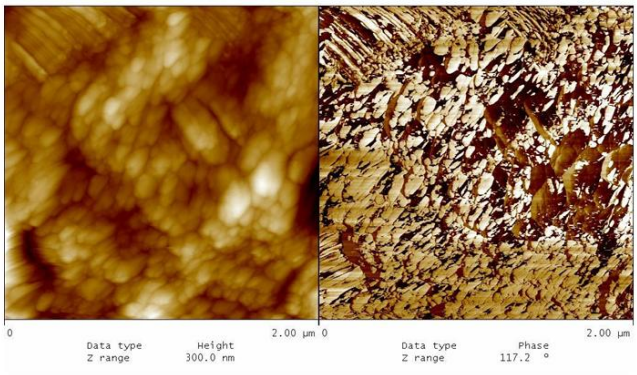
Optical microscopy and AFM were used to investigate the hierarchical structure of "HA"/gelatine nanocomposite gels. In the optical micrograph of Fig.1(a), one can distinguish the outlines of fused gel particles of the order of a few hundreds microns as well as gelatine fibres of 10-20 μm diameter. No HA microparticles can be seen, indicating that the gel is a nanocomposite.

This was followed by AFM studies to perform a comprehensive investigation of the structural hierarchy of the produced gels. AFM was performed in tapping mode at a frequency of 347 kHz. The results included not only surface height maps but also mapping of the phase angle of the viscoelastic surface. Phase angles near zero correspond to elastic materials, such as solids like hydroxyapatite but also air or voids, and larger phase angles correspond to viscoelastic materials, such as gelatine or collagen. In general, it was found that phase angle mapping offered better contrast between the different phases of the nanocomposite than the surface height mapping.

Fig.3 (a) and (b) show the AFM graphs of "HA"/gelatine gels with "HA"/dry gelatine ratio of 0.50 and 0.70, respectively, and Ca/P = 0.43, after being crosslinked in 1% glutaraldeyde for 7h. Fig. 3(a) shows calcified crosslinked gelatine nanofibrils of about 100 nm diameter whereas Fig 3(b) shows much more calcified crosslinked gelatine nanofibrils which have been split down to 50 nm, due to the high calcification.



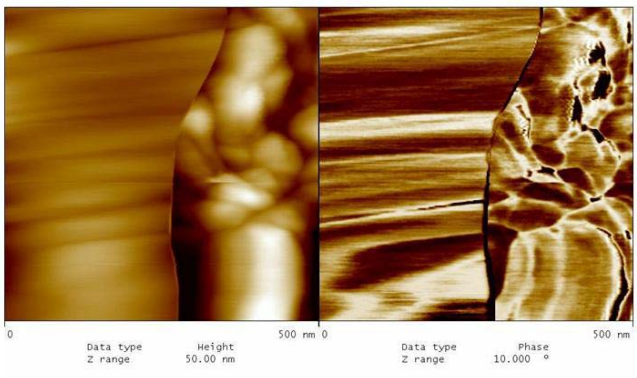
(a)



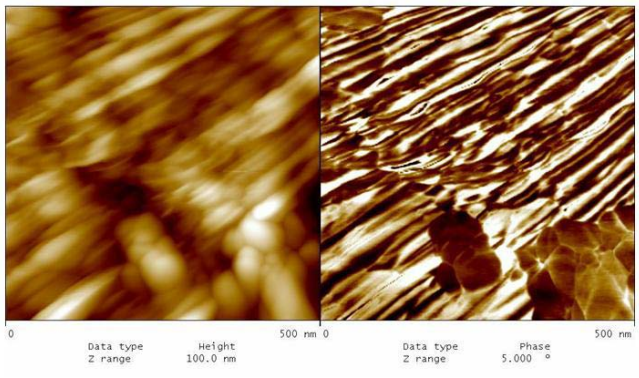
(b)

Fig.3. AFM graphs of "HA"/gelatine gels with Ca/P = 0.43, after being crosslinked in 1% glutaraldeyde for 7 h; samples of 2 x 2 µm area: (a) "HA"/dry gelatine ratio of 0.50 and (b) "HA"/dry gelatine ratio of 0.70.

Fig 4(a) and (b) show AFM graphs of the above "HA"/gelatine gels at higher magnification. The phase angle mapping of Fig. 4(b) shows clearly "hydroxyapatite" nanoparticles of about 60 nm size sitting on a bed of crosslinked gelatine nanofibrils in the gel with "HA"/gelatine = 0.70 g/g, while other smaller "HA" nanoparticles of about 20 nm size are embedded between flat bundles of crosslinked gelatine nanofibrils, where each bundle is a flat bed of nanofibrils of about 45 nm width. This agrees with studies of the bone as the embedded nanoparticles in Fig. 4(b) are of similar dimensions as the HA particles in the natural bone [10-12]. Every 200-300 nm along the flat nanofibril bundles, there are gaps, which have also been detected in the collagen of natural bone [5]. The AFM angle phase mapping of Fig.3(a) shows evidence of D-periodic cross-striated collagen nanofibrils with an average D of 65 nm, which has also been detected in the AFM of natural bone [22]. Fig.4(a) shows "HA" nanoparticles of similar size but fewer as this is the gel with the lower ratio of "HA"/gelatine of 0.50 g/g, splitting the fibre bed in wider bundles of crosslinked gelatine nanofibrils of about 100 nm.



(a)



(b)

Fig.4. AFM graphs of "HA"/gelatine gels with Ca/P = 0.43, after being crosslinked in 1% glutaraldeyde for 7 h; samples of 500x500 nm area: (a) "HA"/dry gelatine ratio of 0.50 and (b) "HA"/dry gelatine ratio of 0.70.

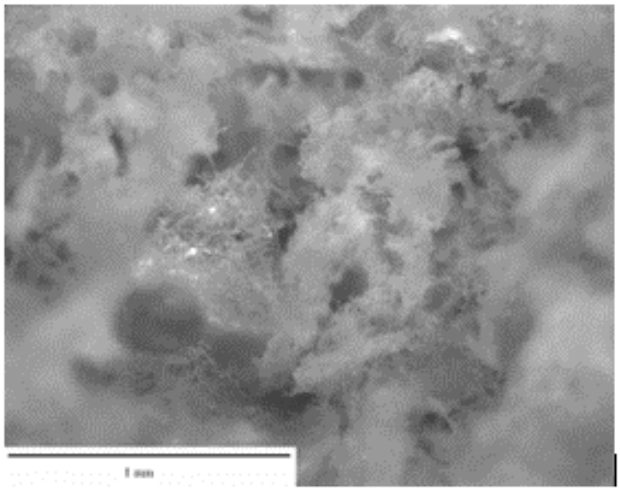
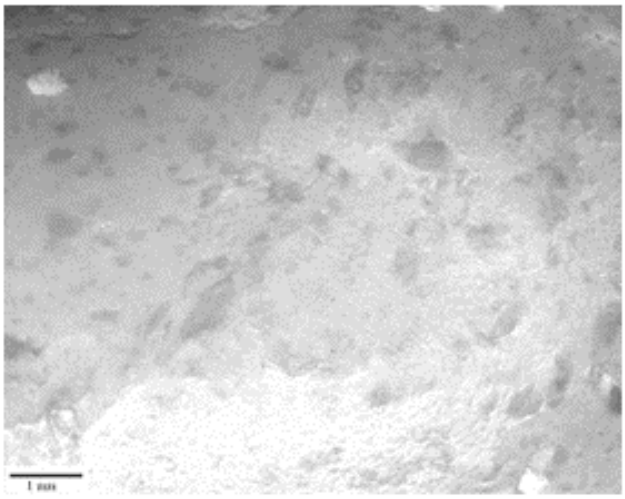


Fig.5. Optical micrograph of a nanocomposite foam with Ca/P = 0.86 and "HA"/gelatine = 0.50 g/g

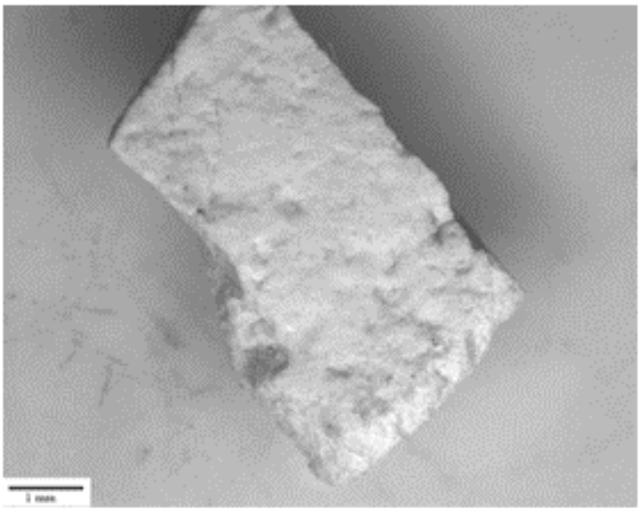
Fig.5 presents an optical micrograph of a foam sample of the nanocomposite foam with the best elastic shear modulus as measured in the DMA tests. The main feature of this foam sample is that it exhibits dual size pores, including large pores of about 100 µm and extremely small pores of 1 µm or less,

whereas the other foam samples presented more uniform porosity.

The produced gels were let to dry slowly in the fridge for 6 months and they lost 92-95% of their weight while they reached the appearance of a compact solid. Fig.6(a) and (b) display optical micrographs for such solids of Ca/P = 0.43 and "HA"/gelatine ratio of 0.50 and 0.70 g/g, respectively. The samples were hard but the sample with "HA"/gelatine = 0.70 was rather brittle.



(a)



(b)

Fig.6. Optical micrographs of "HA"/gelatine compact solid nanocomposites with Ca/P = 0.43, after being crosslinked in 1% glutaraldeyde for 7 h; (a) "HA"/dry gelatine ratio of 0.50 and (b) "HA"/dry gelatine ratio of 0.70.

VI. CONCLUSIONS

In this study a sol-gel process was followed to produce hydroxyapatite/gelatine nanocomposite gels. The gelatine, which is a denatured form of collagen, was dissolved in water at low pH and a type of "hydroxyapatite" (a mixture of different phosphoric calcium salts) was formed in-situ while all ingredients were still in solution. By changing the pH a colloidal suspension was formed which evolved into a nanocomposite gel. The formation of hydroxyapatite nanoparticles rather than microparticles has been targeted in this study to improve the homogeneity and toughness of the resulting composite gels and their suitability as substrates for cell adjustment and proliferation. Crosslinking of the gelatine chains in 1% glutaraldehyde was carried out to stabilize the self-assembled nano- and micro-fibrils so that the produced crosslinked gelatine would resemble collagen. The produced samples were thoroughly rinsed with distilled water to remove any traces of remaining free glutaraldehyde, which would be toxic to any future cell culture.

DMA torsion tests were used to select the optimum crosslinking time in 1% glutaraldehyde, which proved to be 7 h. Gels with different compositions were prepared, specifically with Ca/P molar ratio of 0.43 and 0.86 and "HA"/dry gelatine weight ratio of 0.50 and 0.70 g/g. All gel samples demonstrated viscoelastic behaviour in a frequency range from 1 to 60 Hz and at 37 ^{o}C. The molar ratio of Ca/P = 0.43 proved better, resulting in higher elastic modulus and more intact gel samples. The best gel sample was the sample with Ca/P = 0.43 and "HA"/gelatine = 0.70 g/g, which yielded a maximum elastic shear modulus of 17.6 MPa.

Optical and AFM microscopy of the produced gels revealed that they were indeed nanocomposites of "hydroxyapatite" nanoparticles of about 20 nm, embedded in the nanofibrillar structure of the crosslinked gelatine. In general, the crosslinked gelatine matrix had a structure hierarchy with many features of that of the collagen type I. At micro-level there were microfibrils of 10-20 μm diameter; at nano-level, there were calcified nanofibres of about 100 nm width for "HA"/gelatine = 0.50 g/g and 50 nm width for "HA"/gelatine = 0.70 g/g, indicating that the higher the calcification the more split the nanofibril bundles become. Finally, gaps were detected every 200-300 nm along the flat nanofibril bundles and D-periodic cross-striated nanofibrils as in the collagen of natural bone.

The best foam sample in DMA testing was the nanocomposite with Ca/P = 0.86 and "HA"/gelatine = 0.50 g/g, which had G'= 1.1 MPa and a dual porosity structure, the large pores being most suitable in size for the permeation of osteoblasts. .

REFERENCES

1. Hing KA., Best SM., Tanner KE., Bonfield W. and Revell PA. Biomechanical assessment of bone ingrowth in porous hydroxyapatite. J. of Materials Science-Materials in Medicine 1997; 8(12): 731-736.
2. Yokoyama A., Gelinski M., Kawasaki T., Kohto T., Konig U., Pompe W. and Watari F. Biomimetic porous scaffolds with high elasticity made from mineralized collagen-An animal study. J. of

Biomedical Materials Research Part B-Applied Biomaterials 2005; 75B(2): 464-472.
3. Grimes JS., Bocklage TJ and Pitcher JD. Collagen and biphasic calcium phosphate bone graft in large osseous defects. Orthopedics 2006; 29(2): 145-148.
4. New tissue engineered bone technology to revolutionise reconstructive surgery. www.isotis.com
5. Rho J., Kuhn-Spearing L. and Zioupos P. Mechanical properties and the hierarchical structure of the bone. Medical Engineering and Physics. 1998; 20.
6. Carter DR., and Hayes W. The compressive behaviour of bone as a two-phase porous structure. J.Bone Jt Surg. 1977; 59A: 954-962.
7. Gibson LJ. The mechanical behaviour of cancellous bone. J of Biomechanics 1985; 18: 317-328.
8. Brodsky B. and Ramshaw JA. The collagen triple-helix structure. Matrix Biol. 1997; 15: 545-554.
9. Mizuno K. and Bachinger HP. Thermal stability and folding of collagen triple helices. Connective Tissue. 2003; 35: 207-217.
10. Glimcher M. Mechanisms of calcification: role of collagen fibrils and collagen-phosphoprotein complexes in vitro and in vivo. Anat. Rec. 1989; 224: 139-153.
11. Ziv V. and Weiner S. Bone crystal size: a comparison of transmission electron microscopic and x-ray diffraction line-width-broadening techniques. Conn. Tissue Res. 1994; 30: 165-175.
12. Landis WJ. The strength of calcified tissue depends in part on the molecular structure and organization of constituent mineral crystals in their organic matrix. Bone 1995; 16: 533-544.
13. James C-B. and Uhl TL. A review of articular carticular pathology and the use of glucosamine sulfate. J. Athl. Train. 2001; 36(4): 413-419.
14. Reilly DT., Burstein AH. And Frankel VH., Elastic modulus for bone. J. Biomech. 1974; 7(3): 271-281.
15. Zhai Y., Cui FZ. And Wang Y. Formation of nano-hydroxyapatite on recombinant human-like collagen fibrils. Current Appl. Physics 2005; 5(5): 429-432.
16. Lin XY, Fan HS., Li XD., Tang M., Zhang LL., Tan YF., Xu JR. and Zhang XD. In vitro and in vivo biocompatibility of nano-hydroxyapatite/collagen composites. Advanced Biomaterials VI Key Engineering Materials 2005; 288-289: 195-198.
17. Kim HW., Yoon BH. And Kim HE. Microsphere of apatite-gelatin nanocomposite as bone regenerative filler. J. Materials Sci.-Materials in Medicine 2005; 16(12): 1105-1109.
18. Kikuchi M., Ikoma T., Syoji D., Matsumoto HN., Koyama Y., Itoh S., Takakuda K., Shinomiya K. and Tanaka J. Porous body preparation of hydroxyapatite/collagen nanocomposites for bone tissue regeneration. Bioceramics 2004; Vol 16 Key Engineering Materials; 254-2: 561-564.
19. Kim HW., Song JH. And Kim HE. Nanofibre generation of gelatin-hydroxyapatite biomimetics for guided tissue regeneration. Advanced Functional Materoials; 2005; 15(12): 1988-1994.
20. Kim HW., Kim HE. And Salih V. Stimulation of osteoblast responses to biomimetic nanocomposites of gelatin-hydroxyapatite for tissue engineering scaffolds. Biomaterials 2005; 26(25): 5221-5230.
21. Itoh S., Kituchi M., Takuda K., Nagaoka K., Koyama Y., Tanaka J. and Shinomiya K. Implantation study of a novel hydroxyapatit/collagen composite into weight-bearing sites of dogs, J. Biomed. Mater. Res. 2002; 63: 507-515.
22. Bozek L., de Groot J., Odlyha M., Nicholls B., Nesbitt S., Flanagan A. and Horton M. Atomic force microscopy of collagen structure in bone and dentine revealed by osteoclastic resorption. Ultramicroscopy 2005; 105: 79-89.