**STRUCTURAL HIERARCHY OF BIOMIMETIC MATERIALS FOR
TISSUE ENGINEERED VASCULAR AND ORTHOPAEDIC GRAFTS**

C.Lekakou^a, D.Lamprou^b, U.Vidhyarthi^b, E.Karopoulou^a and P.Zhdan^a

^aCentre for Materials, Surfaces and Structural Systems

^bCentre for Biomedical Engineering

School of Engineering

University of Surrey

Guildford

Surrey GU2 7XH

Abstract

Gelatine gels and gelatine/elastin gels have been prepared to be used in tissue engineered vascular grafts. Optical microscopy and AFM revealed that the gelatine formed nanofibrils as in soft collagen tissues. The gelatine/elastin gels were nanocomposites with flat elastin nanodomains embedded in the gelatine matrix mimicking the structure of the tunica media in arteries. Gelatine/"hydroxyapatite" nanocomposites were prepared with the in situ production of "hydroxyapatite" in solution. AFM revealed "hydroxyapatite" solid nanoparticles of about 20 nm size embedded in the gelatine matrix which formed a hierarchical structure similar to that of the collagen matrix in bone. The application of a magnetic field of 9.4 T resulted in the elongation and orientation of gelatine particles and orientation of gelatine microfibrils in a direction perpendicular to that of the magnetic field.

1. INTRODUCTION

Collagen is the major constituent of both tissue and bone types of organs and, for this reason, it has been selected as the main matrix constituent in the biomimetic materials developed in this study. Collagen fibres exist at different scales, from nano- to micro-levels, and have adopted different types of orientation in single or multiple layer formation in tissue, cartilage and bone.

Arteries generally consist of three layers [1]: the tunica intima, media and adventitia. In these, collagen fibres are generally oriented in the axial direction. A lining of endothelial cells, also oriented in the axial direction, is in contact with the blood flow in tunica intima. Elastin plates are embedded between collagen fibres, mainly in tunica media which constitutes the major part of the artery wall. Tunica media also includes smooth muscle cells, embedded in the collagen/elastin matrix and oriented in the circumferential direction. The elastin/collagen ratio varies from 0.25 to 1.5, depending on the type of artery [2]. Collagen is stiffer with a Young's modulus in the range of 5 to 10 MPa, whereas elastin has a Young's modulus of 0.4 – 1 MPa and can undergo a stretch ratio of 1.6 [3-8].

Cortical bone is made from long, cylindrical osteons of about 10-500 μm diameter, each [9]. Each osteon is a cylinder consisting of multiple layers wrapped around each other of about 3-7 μm thickness for each layer [10]. Each layer consists of collagen micro-fibres oriented in the same direction for each layer, e.g. 90 °, 45 °, 0 ° [9, 11]. Each collagen fibre is really a bundle of micro-fibrils of about 0.5 μm diameter each [9]. Each micro-fibril consists of lamellae of triple helix collagen molecules, with gaps in-plane between these lamellae in which

hydroxyapatite crystals of about 50 x 25 x 2 nm are embedded [12-14]. Cortical bone contains about 60% hydroxyapatite, 16% collagen fibres, 23% water and 2% ground substance [9].

At molecular level, collagen is composed of three protein chains, wound together in a triple helix of about 300 nm length and 1.5 nm diameter for type I collagen [15]. Type I collagen molecules form D-periodic cross-striated fibrils (axial periodicity $D = 67$ nm), providing the major biomechanical scaffold for cell attachment and anchorage of other macromolecules [15, 16].

Hence, one of the tasks in the creation of biomimetic materials, as envisaged in this study, is the reproduction and characterisation of this type of orientation in collagen or other related biomimetic materials. Even in recent attempts for tissue engineered tubular vascular grafts [17, 18], no effort has been put into orienting the collagen fibres and microfibrils. Tranquillo et al [19] found that application of a magnetic field of 4.7 T induced collagen orientation resulting in a birefringence $\Delta n = 12.2 \pm 2.9 \times 10^{-6}$.

Elastin has been introduced in collagen/elastin based tissue engineered vascular grafts [20] either in gel-derived fibrous products [21] or electrospun micro- and nano-fibrous scaffolds [22,23]. Apart from the purpose of mimicking the artery composition, elastin has been reported to improve the mechanical properties of collagen/elastin scaffolds which would have been too weak to be implanted as vascular grafts if they were based only on collagen [24].

Although the major part of tissue engineering studies so far have been based on cell culture and proliferation in scaffolds, it has been possible to embed cells in-situ during the generation of three-dimensional gels [25]. Gel structures are expected to mimic much better tissue and bone materials. In many recent studies for orthopaedic grafts [26-30], gelatine has been explored instead of collagen, possibly due to the high costs of soluble collagen. Gelatine is a denatured form of collagen, consists of amino-acid chains and is soluble in water [31].

Different types of gelatine/hydroxyapatite(HA) or collagen/hydroxyapatite composites have been proposed for orthopaedic grafts. Scaffolds made from nanocomposite fibres with gelatine as the matrix and HA nanocrystals showed improved bone-derived cellular activity compared to pure gelatine scaffolds [26], and better attachment and proliferation of osteoblasts than conventional microcomposite gelatine/HA scaffolds [32]. Hydroxyapatite seems to promote osteoconduction [33], growth of human mesenchymal stem cells and osteogenic differentiation [34]. Du et al [35] concluded that HA/collagen nanocomposites are the ideal bone-mimic materials, following a process of implant degradation and bone substitution in vivo which was reminiscent of bone modelling. Zhang et al [36] used TEM to study the self-assembly of mineralised collagen nanofibrils: they found self-assembled, unmineralised collagen nanofibrils of about 4 nm diameter and mineralised collagen nanofibrils of 5.5-6.9 nm diameter, where the difference was attributed to the HA crystal layer on the surface of the fibrils with the HA crystallographic c-axis preferentially aligned along the collagen fibril axis.

The rationale behind this study was to prepare collagen, collagen/elastin and collagen/HA nanocomposites which mimic the structure of arteries and bone as closely as possible and could be used in tissue engineering. Gelatine has been used as a low-cost source of soluble collagen. After the desired structure was achieved, the gelatine chains were crosslinked in glutaraldehyde. The architectural hierarchy in the produced composites was studied in optical microscopy and atomic force microscopy (AFM).

2. MATERIALS AND METHODS

2.1 GELATINE FIBRIL ORIENTATION USING A MAGNETIC FIELD

800 mg of powder of animal gelatine was dissolved in 40 ml 0.01 N acetic acid under stirring with a magnetic stirrer. The solution was split into three vials. Vial 1 was placed in an MRI electromagnetic field of 9.4 T in the axial direction for 10 min (in case that the electromagnetic field produces any molecular orientation of the gelatine molecules in solution), then it was removed to add 0.1 N NaOH until pH=12 (this results in a dispersion and eventually precipitation of gelatine particles, see Fig.1), and the vial was put back into the MRI field for 10 min (with the aim to elongate and orientate the gelatine particles and to orientate the gelatine fibrils and molecular chains). In Vial 2 no electromagnetic field was applied to the solution, 0.1 N NaOH was added just before the vial was placed in the MRI field, and Vial 2 was left in the MRI field for 10 min (to investigate whether there would be any differences between this and the procedure followed in Vial 1). In Vial 3 the same procedure was followed as in Vial 2 but it was left in the MRI field for 15 min to investigate whether a longer time under the electromagnetic

field during the stage of precipitation of gelatine particles would generate more orientation . Then all vials were placed in cold storage for 18 h for gelation to take place and were subsequently examined under an optical microscope. Fig.2 shows that the magnetic field produces a trend in circumferential orientation (orientation normal to the direction of the magnetic field), especially in Vial 3 where the magnetic field had the longest effect just during the precipitation of gelatine particles: in this the gelatine particles are elongated (about 1 mm length and 500 μm lateral dimensions) and oriented in the orientation direction as well as the gelatine fibrils of about 10 to 20 μm diameter (see Fig.2 and 3). The application of the magnetic field during the solution stage (before the addition of 0.1 N NaOH) in Vial 1 in Fig.2 does not seem to have resulted in any better orientation than in Vial 2, so it has been concluded that the critical step is the application of the magnetic field during the stage of precipitation of the gelatine particles for sufficiently long time to generate a good and permanent orientation that is not going to relax back once the vial is removed from the MRI field.

This part has included preliminary investigations on the application of the magnetic field and its effects on particle and fibril orientation in gelatine gels and has not been extended to gelatine/elastin and gelatine/"hydroxyapatite" composites yet. Furthermore, the results and micro- and nanostructural studies of the following sections in this paper do not involve the application of magnetic field.

2.2 MATERIALS AND PREPARATION METHODS OF COMPOSITES FOR VASCULAR GRAFTS

The main materials were powder of animal gelatine (soluble in water in acidic environment and derived from collagen) and powder of E1625 elastin (insoluble) from bovine neck ligament from Sigma-Aldrich. Gelatine gels and 70/30 wt. gelatine/elastin gels were prepared as follows: 160 mg gelatine for the gelatine gels or 112 mg gelatine for the gelatine/elastin gels was dissolved in 20 ml 0.01N acetic acid under stirring with a magnetic stirrer. Elastin was added under stirring to prepare an elastin dispersion in gelatine solution for the production of gelatine/elastin gels. 0.01 N NaOH was added under stirring until pH=12 and the dispersions (Fig.1) were placed in cold store for 15 min. This resulted in the precipitation of gelatine particles, which would co-precipitate with elastin particles where appropriate. Then, the clear liquid above the precipitates was removed with a micro-pipette and the precipitates were placed on aluminium discs and moved to cold store for 18 h to form gels (see Fig.3). Finally the gels were placed in 1% glutaraldehyde for 8 h, which proved to be the optimum time for the crosslinking of collagen chains [37].

2.3 MATERIALS AND PREPARATION METHODS OF COMPOSITES FOR ORTHOPAEDIC GRAFTS

The matrix material was produced from powder of animal gelatine (soluble, derived from collagen). The aim was to produce a dispersed phase as nanoparticles of a form of CaP salts, denoted here as “hydroxyapatite” (“HA” abbreviation), in situ from the reaction of $\text{Ca}(\text{OH})_2$ and H_3PO_4 . This reaction may produce different types of salts, such as $\text{Ca}_3(\text{PO}_4)_2$ if the Ca/P molar ratio is higher

than 1.67 or CaHPO_4 otherwise [38]. Since the former has been reported to lead to the production of mechanically weak scaffolds [39], a molar ratio $\text{Ca/P} = 0.43$ was used in this study.

0.8 g of gelatine powder was dissolved in 9.552 ml of 1 M H_3PO_4 (A g) under stirring with a magnetic stirrer. 4A g of 0.1 M $\text{Ca}(\text{OH})_2$ was added to the gelatine solution under stirring and distilled water was also added to make a total volume of 160 ml solution. When the pH was changed to 9 by adding 1 M NaOH, a dispersion of gelatine and “hydroxyapatite” was formed. The dispersion was left to rest for 18 h to form a precipitate of gelatine and “hydroxyapatite” particles. The precipitate was filtrated, placed on aluminium discs and was left in cold store for 15 h to gel. The gels were placed in 1% glutaraldehyde for 7 h which proved to be the optimum time for crosslinking of the gelatine chains in gelatine/”hydroxyapatite” gels [40].

3. RESULTS

3.1 AFM OF GELATINE AND GELATINE/ELASTIN COMPOSITES FOR VASCULAR GRAFTS

AFM was employed to investigate the nanostructure of the produced gels for vascular grafts. Fig.4 presents the surface height mapping of a gelatine gel sample of an area of 356x356 nm and Fig.4(b) displays a height analysis along a defined line. Using NanoScope software, version 5.12, by Digital Instruments (Veeco), 10 equally spaced lines of section analysis (as in Fig. 4(b)) were drawn for the sample in Fig.4, all lines perpendicular to the main fibril axis. After collecting all

data of height and fibril width analysis, it was concluded that the gel displays the characteristic nanostructure of collagen with nanofibrils of diameter in the range of 1.5 to 7 nm, which means they contain bundles of 1 to 5 triple helices of protein chains. All nanofibrils are homogeneously laid in the AFM image and oriented parallel to each other, as is the case of collagen in tissues.

A 70/30 collagen/elastin composition would mimic the composition of thoracic, iliac and femoral arteries [2]. AFM in tapping mode was used to study the sample and results were obtained for both surface height mapping to study the morphology and mapping of the phase angle, δ , of the viscoelastic surface; the latter may be used in the case of blends to distinguish between two materials with differences in the viscoelastic properties, where $\tan\delta$ is the ratio of the loss modulus divided by the elastic modulus. Fig.5 presents the AFM surface height mapping of a 70/30 wt. gelatine/elastin gel of an area of 659x659 nm: it is clear that the gel is a nanocomposite of flat domains of 200 nm width and length and about 50 nm thickness dispersed in a fibrous matrix. The corresponding phase angle map in Fig. 5 shows clearly that these flat domains are of a different material from that of the matrix, and given the blend composition, it can be concluded that the flat nanodomains are made of elastin (sometimes covered by gelatine at their central part on the micrographs) and they lie embedded in the gelatine matrix. The gelatine has a nano-structure of 50 nm wide bundles of nanofibrils, created by the embedment of the elastin nanodomains, which did not exist in the pure gelatine nanostructure (see Fig.4). Fig. 6 shows an AFM image of the 70/30 gelatine/elastin gel of an area of 199x199 nm, which apart from the elastin nanodomains reveals smaller features in the hierarchy of the gelatine

matrix nanostructure, including a bed of gelatine nanofibrils of up to 7 nm diameter, which form wider flat bundles of aligned nanofibrils (of about 50 nm width for each bundle) due to the embedment of elastin nanodomains.

3.2 OPTICAL MICROSCOPY AND AFM OF GELATINE/"HA" COMPOSITES FOR ORTHOPAEDIC GRAFTS

Fig.7 shows an optical micrograph of the produced gelatine/"HA" gel. The outlines of fused gel particles of the order of a few hundreds microns can be seen as well as gelatine fibres of about 10-20 μm diameter. No opaque particles (which might have been "hydroxyapatite" microparticles) could be seen on the optical microscope under any magnification, indicating that there were no opaque particles at microlevel. On the other hand, if in the preparation procedure the pH was changed to 9 before 0.1 M $\text{Ca}(\text{OH})_2$ was added, a microcomposite gelatine/"HA" gel was formed in which "HA" microparticles can be easily seen in the optical micrograph of Fig.8. "HA" microparticles made the microcomposite gel very frail and fragile, easy to fall apart into small pieces, in contrast with the gel in Fig.7 (thought to be a gelatine/"HA" nanocomposite) which could remain intact and strong when handled.

A series of AFM images were taken at different magnifications to investigate the structural hierarchy of the gelatine/"HA" nanocomposite gel. In this case the AFM images included not only the surface height mapping but also mapping of the phase angle of the viscoelastic surface. Phase angles close to 0 would correspond to elastic materials, like solid "HA" particles, air or voids, whereas larger phase angles indicate viscoelasticity present in the gelatine matrix. It was found that the

phase angle mapping demonstrated contrast between the gelatine and “HA” phases and it was more useful to identify the “HA” particles than the surface height mapping.

Fig.9(a) demonstrates that whereas the surface height map shows short gelatine fibres of about 400 nm diameter, the phase angle graph shows a much clearer and more refined picture of gelatine fibres of about 100 nm diameter, possibly calcified. The fibres lose continuity with “holes” every 200-300 nm along their length which represents gaps of similar periodicity in the collagen of bone [9]. Fig.9(b) presents regions (e.g. circled region) where D-period cross-striated nanofibrils are easily found with a periodicity $D = 65$ nm. Fig.9(c) indicates that there are dark elastic regions in the phase angle mapping which do not correspond to valleys in the surface height mapping and, hence, are solid “HA” particles rather than voids. Fig.9(d) shows “HA” particles of about 60 nm diameter sitting on a bed of gelatine nanofibrils and other smaller “HA” nanoparticles of about 20 nm size embedded between flat bundles of gelatine nanofibrils, where each bundle is a flat bed of nanofibrils of about 45 nm width. This mirrors the structure of the gelatine/elastin nanocomposites in Fig.5, which leads to the conclusion that nanodomains of either “hydroxyapatite” or elastin embedded in the gelatine structure separate the gelatine nanofibrils into flat bundles. The embedded “HA” nanoparticles are of similar dimensions as the hydroxyapatite particles in the bone [12, 14].

4. DISCUSSION

This has been a study of the structural hierarchy of biomimetic materials for soft and hard tissues. The starting point was that collagen is the matrix of all these materials in organisms which was substituted by gelatine in this paper, due to its much lower cost while gelatine still consists of fragments of soluble collagen. The structure hierarchy of the produced gels was investigated using optical microscopy and AFM. The first step was to examine the structure and alignment in produced gelatine gels and compare it with the expected collagen structure in organisms, without the complication of a second phase (such as elastin or hydroxyapatite) in the gels. This was followed by the successful preparation of nanocomposite gels, involving either a soft second phase, i.e. elastin, or a hard second phase, i.e. CaP particles denoted as “hydroxyapatite”. The aim of producing nanocomposites (rather than microcomposites) was important in both cases from the point of structural and mechanical integrity and cell culture. The next question was how the nanodomains were dispersed in each type of nanocomposite (for soft or hard tissue applications, respectively) and how they affected the structure of the gelatine matrix and the alignment of nanofibrils at nanolevel. The investigation for possible gaps and voids in the structure in the self-assembly of nanofibrils and embedment of the second phase is also important for the potential adherence and propagation of cells.

Gelatine gels contained homogeneously laid, parallel oriented nanofibrils of 1.5 to 7 nm diameter that must have been made of about 1 to 5 triple helices of protein chains, resembling the nanostructure of collagen in tissues [15]. 70/30 wt. gelatine/elastin gels were prepared to mimic the composition of thoracic iliac and

femoral arteries. AFM demonstrated that the nanocomposite gels also resembled the structure of the tunica media of these arteries [1], with flat elastin nanodomains of 200 x 200 x 50 nm embedded within gelatine so that they split the bed of 1.5-7 nm diameter nanofibrils into flat bundles of about 50 nm width. The produced gaps between the nanofibril bundles are of the order of 10 nm width, which seems very small to have any negative effects on the mechanical integrity of the grafts but introduce additional roughness at nanolevel that may be beneficial for the adherence of cells.

It was also possible to prepare gelatine/"hydroxyapatite" nanocomposites from the in situ reaction of $\text{Ca}(\text{OH})_2$ and H_3PO_4 in a gelatine solution and subsequent formation of a colloidal dispersion of gelatine and "hydroxyapatite", which eventually resulted in a precipitate of gelatine particles and "hydroxyapatite" nanoparticles that would be embedded between gelatine nanofibrils in the gel formation. AFM demonstrated that "HA" nanoparticles of about 20 nm were embedded within the gelatine structure so that they split the bed of gelatine nanofibrils into flat bundles of about 45 nm width. HA crystals of similar size are found in natural bone [12-14]. Gaps were detected every 200-300 nm along these flat bundles which are also found in the collagen of natural bone [9]. D-periodic cross-striated collagen nanofibrils were detected in the AFM phase angle mapping images with an average $D = 65$ nm which has also been found in the AFM of natural bone [41].

All these types of gaps identified in the produced nanocomposite gel and their similarities to those in the bone are important as potential sites of anchorage for

cells and macromolecules in the organism. It is clear that the composite gels for either the vascular or the orthopaedic applications contain more and larger such gaps than the pure gelatine gels, due to the embedment of the second dispersed phase (either soft or hard phase) and the bundling of the nanofibrils of the matrix; this increases the nanoroughness and the number of anchorage sites.

All gels produced in this study displayed evidence of self-assembly of aligned gelatine nanofibrils at nanolevel, with some deviation in nanocomposites due to the embedding of the second dispersed phase. On the assumption of formation of CaHPO_4 in the gelatine/"HA" nanocomposites in this study [38] (given that the employed Ca/P ratio was less than 1.67), the produced gels contained 41% "HA" in the "HA"/dry gelatine blend (i.e. the water of the gel has not been included) that led to gels of good mechanical integrity, with the potential to be used in electrospinning. Kim et al [26] produced gelatine/HA nanocomposite gel scaffolds by electrospinning, with 20 to 40% HA. Their TEM images showed HA needle crystals and gelatine nanofibrils in each electrospun gel fibre where the matrix had a much more random form of gelatine nanofibrils than in the present study and the HA needles were oriented in a totally random manner, causing discontinuities in the electrospun fibres. On the contrary, the gelatine nanofibrils and nanofibril bundles display much better alignment in the present study at nanolevel (Fig.9(d)) and any solid nanoparticles are either embedded between the aligned nanofibrils with solid regions aligned in the main alignment axis or are in the form of approximately spherical nanoparticles of about 60 nm in diameter, bringing them much closer to the bone nanostructure..

The application of a magnetic field of 9.4 T during the precipitation stage of gelatine particles for 15 min induced elongation and orientation of the fused gelatine microparticles and the gelatine microfibrils in a direction perpendicular to that of the magnetic field.

5. REFERENCES

1. Levick JR. An introduction to cardiovascular physiology. 3rd Edition. Oxford: Oxford Univ. Press Inc; 2000.
2. Hastings GV. Cardiovascular biomaterials. London: Springer-Verlag; 1992.
3. O'Rourke MF. Arterial function in health and disease. Edinburgh: Churchill Livingstone; 1982.
4. Brooker CG. Human structure and function. 2nd Edition. Nursing applications in clinical practice. St Louis: Mosby; 1998.
5. Marieb EN. Human anatomy and physiology. 3rd Edition. The Benjamin/Cummings series in the Life Sciences. Redwood City, California: The Benjamin/Cummings Publishing Company, Inc ; 1995.
6. Solomon EP. Human anatomy and physiology. 2nd Edition. London Philadelphia (PA): Saunders College; 1990.
7. Tortora GJ, Grabowski SR. Principles of anatomy and physiology. 8th Edition, New York: HarperCollins College; 1996.
8. Ilona BD. Structure and function of elastin and collagen. Budapest: Akademiai Kiado; 1966.

9. Rho J, Kuhn-Spearing L,, Zioupos P. Mechanical properties and the hierarchical structure of bone. *Medical Engineering & Physics* 1998; 20(2): 92-102.
10. Marroti G. A new theory of bone lamination. *Calcif Tissue Intern.* 1993; 53(1): S47-56.
11. Giraud-Guille MM. Twisted plywood architecture of collagen fibres in human compact osteons. *Calcif Tissue Intern.* 1988; 42: 167-180.
12. Glimcher M. Mechanisms of calcification: role of collagen fibrils and collagen-phosphoprotein complexes in vitro and in vivo. *Anat. Rec.* 1989; 224: 139-153.
13. Ziv V, 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.
14. Landis WJ. The strength of calcified tissue depends in part on the molecular structure and organisation of its constituent mineral crystals in its organic matrix. *Bone* 1995; 16: 533-544.
15. Brodsky B, Ramshaw JA. The collagen triple-helix structure. *Matrix Biol.* 1997; 15: 545-554.
16. Mizuno K, Bachinger HP. Thermal stability and folding of collagen triple helices. *Connective Tissue.* 2003; 35: 207-217.
17. Berglund J.D, Mohseni MM, Nerem RM, Sambanis A. A biological hybrid model for collagen-based tissue engineered vascular grafts. *Biomaterials* 2003; 24: 1241-1254.
18. Baguneid M, Murray D, Salacinski HJ, Fuller B, Hamilton G, Walker M., Seifalian AM. Shear-stress preconditioning and tissue-engineering-based

- paradigms for generating arterial substitutes. *Biotechnol. Appl. Biochem.* 2004; 39: 151-157.
19. Tranquillo RT, Girton TS, Bromberek BA, Tribes TG, Mooradian DL. Magnetically orientated tissue-equivalent tubes: application to a circumferentially orientated media-equivalent. *Biomaterials* 1996; 17: 349-357.
 20. Daamen WF, Van Moerkerk HTB, Hafmans T, Buttafoco L, Poot AA, Veerkamp JH, Van Kuppevelt TH. Preparation and evaluation of molecularly-defined collagen-elastin-glycosaminoglycan scaffolds for tissue engineering. *Biomaterials* 2003; 24(22): 4001-4009.
 21. Buijtenhuijs P, Buttafoco L, Poot AA, Daamen WF, Van Kuppevelt TH, Dijkstra PJ, De Vos RAI., Sterk LMT, Geelkerken BRH, Feijen J, Vermes I. Tissue engineering of blood vessels: characterisation of smooth-muscle cells for culturing on collagen-and-elastin-based scaffolds. *Biotechn. And Appl. Biochem.* 2004; 39: 141-149.
 22. Boland ED, Matthews JA, Pawlowski KJ, Simpson DG, Wnek GE, Bowlin GL. Electrospinning collagen and elastin: preliminary vascular tissue engineering. *Frontiers in Bioscience* 2004; 9: 1422-1432.
 23. Buttafoco L, Kolkman NG, Engbers-Buijtenhuijs P, Poot AA, Dijkstra PJ, Vermes I, Feijen J. Electrospinning of collagen and elastin for tissue engineering applications. *Biomaterials* 2006; 27(5): 724-734.
 24. Berglund JD, Nerem RM, Sambanis A. Incorporation of intact elastin scaffolds in tissue-engineered collagen-based vascular grafts. *Tissue Engineering* 2004; 10(9-10): 1526-1535.

25. Mironov V, Kasyanov V, Shu XZ, Eisenberg C, Eisenberg L, Gonda S, Trusk T, Markwald RR, Prestwich GD. Fabrication of tubular tissue constructs by centrifugal casting of cells suspended in an in-situ crosslinkable hyaluronan-gelatin hydrogel. *Biomaterials* 2005; 26: 7628-7635.
26. Kim HW, Song JH, Kim HE. Nanofiber generation of gelatin-hydroxyapatite biomimetics for guided tissue regeneration. *Advanced Functional Materials* 2005; 15(12): 1988-1994.
27. Kim HW, Yoon BH, Kim HE. Microsphere of apatite-gelatin nanocomposite as bone regenerative filler. *J. of Materials Science-Materials in Medicine* 2005; 16(12): 1105-1109.
28. Askarzadeh K, Orang F, Moztarzadeh F. Fabrication and characterisation of a porous composite scaffold based on gelatine and hydroxyapatite for bone tissue engineering. *Iranian Polymer J.* 2005; 14(6): 511-520.
29. Teng SH, Shi JJ, Wang Y, Peng BX, Chen LJ. Effects of alpha component content of gelatins on mechanical properties of gelatine/hydroxyapatite composites. *Acta Polymerica Sinica* 2006; 1: 65-69.
30. Furuichi K, Oaki Y, Imai H. Preparation of nanotextured and nanofibrous hydroxyapatite through dicalcium phosphate with gelatine. *Chem. Of Materials* 2006; 18(1): 229-234.
31. Word AG, Courts A. The science and technology of gelatine. London: Academic Press; 1977.
32. Kim HW, Kim HE, Salih V. Stimulation of osteoblast responses to biomimetic nanocomposites of gelatine-hydroxyapatite for tissue engineering scaffolds. *Biomaterials* 2005; 26(25): 5221-5230.

33. Allegrini S, Rumpel E, Kauschke E, Fanghanel J, Konig B. Hydroxyapatite grafting promotes new bone formation and osseointegration of smooth titanium implants. *Annals of Anatomy-Anatomischer Anzeiger* 2006; 188(2): 143-151.
34. Zhao F, Grayson WL, Ma T, Bunnell B, Lu WW. Effects of hydroxyapatite in 3-D chitosan-gelatin polymer network on human mesenchymal stem cell construct development. *Biomaterials* 2006; 27(9): 1859-1867.
35. Du C, Cui FZ, Feng QL, Zhu XD, De Groot K. Tissue response to nano-hydroxyapatite/collagen composite implants in marrow cavity. *J. of Biomedical Materials Research* 1998; 42(4) 540-548.
36. Zhang W, Liao SS, Cui FZ. Hierarchical self-assembly of nano-fibrils in mineralised collagen. *Chemistry of Materials* 2003; 15(16): 3221-3226.
37. Lekakou C. Biomimetic materials for tissue engineered vascular and orthopaedic grafts, presented at the British Soc. Rheology (BSR) 2006 Annual Meeting. *Dynamics of Biofluids and Biomaterials*, Manchester, December 11-12, 2006
38. Suchanek W, Yoshimura M. Processing and properties of hydroxyapatite-based biomaterials for use as hard tissue replacement implants. *J. Mater. Res.* 1998; 13(1): 94-117.
39. Itoh S, Kikuchi M, Takakuda K, Nagaoka K, Koyama Y, Tanaka J, Shinomiya K. Implantation study of a novel hydroxyapatite/collagen composite into weight-bearing sites of dogs. *J. Biomed. Mater. Res. Appl. Biomaterials.* 2002; 63: 507-515.
40. Vidyarthi U. Bone Tissue Engineering. MSc Thesis. University of Surrey. Guildford UK; 2005.

41. Bozec L, de Groot J, Odlyha M, Nicholls B, Nesbitt S, Flanagan A, Horton M.

Atomic force microscopy of collagen structure in bone and dentine revealed by osteoclastic resorption. *Ultramicroscopy* 2005; 105: 79-89.

Figures

Fig.1 Suspension of gelatine particles formed by adding 0.1 NaOH in a gelatine solution of 0.01 N acetic acid.

Fig.2. Optical micrographs of gels generated under the influence of a magnetic field of 9.4 T in the axial direction (horizontal direction in the micrographs).

Fig.3. Optical micrograph showing the microfibrillar structure of a gelatine gel.

(a)

(b)

Fig.4. AFM of gelatine gel for vascular grafts. (a) surface height mapping (b) image analysis along indicated line.

Fig.5. AFM of 70/30 gelatine/elastin gel for vascular grafts; surface height and phase angle maps of an area of 659x659 nm.

Fig.6. AFM of 70/30 gelatine/elastin gel for vascular grafts; surface height mapping of an area of 199x199 nm and image analysis along indicated line.

Fig. 7. Optical micrograph of a gelatine/”HA” nanocomposite gel prepared according to the procedure described in section 3.1

Fig. 8. Optical micrograph of a gelatine/”HA” microcomposite gel during the preparation of which the pH was changed to 9 before 0.1 M $\text{Ca}(\text{OH})_2$ was added.

(a)

Fig 9(a)

(b)

Fig 9b

(c)

Fig.9(c)

(d)

Fig.9d

Fig.9. AFM surface height and phase angle mappings of gelatine/"HA" nanocomposite gel at different magnifications.