THE ROLE OF THREE-DIMENSIONAL SCAFFOLDS IN THE REGENERATION OF JOINT CARTILAGE

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Abstract: A variety of polymer scaffolds with pore architecture consisting of interconnected spherical pores with the same architecture but varying mechanical properties (in particular elastic modulus), water sorption capacity, pore surface characteristics (surface tension, presence of hydrophilic groups or electric charges) was prepared and implanted in a 3mm diameter full thickness defects in the knee joint cartilage of rabbits in order to show the influence of the scaffold properties on the histological characteristics of the regenerated tissue.

1 INTRODUCTION

The poor self-regeneration of articular cartilage has stimulated continued efforts to develop tissue engineering techniques (Patrick 1998, Shoichet 1998). The clinical practice focused on either transplantation or implantation of autologous chondrocytes, a tissue engineering technique started in 1987 and since then employed in the treatment of around 10,000 patients all over the world (Brittberg 2003, Smith 2005, Marlovits 2006). In this technique tissue regeneration is expected to be originated in adult chondrocytes transplanted to the site of the defect. Previously to the transplant to the site of the defect chondrocytes must be expanded "in vitro" to the cell numbers necessary to produce sufficient extracellular matrix, since no further mitosis is expected "in vivo". "In vitro" culture has been shown to de-differentiate cells, that loose the phenotype of hyaline cartilage chondrocytes and start expressing proteins such as type I collagen, characteristic of other connective tissues. On the other hand, microfracture of the subchondral bone allows the recruitment of mesenchymal stem cells from subchondral bone marrow whose differentiation to chondrocytes is another way of possible regeneration (Stradman 1999).

Whatever the cell source for regeneration, the correct differentiation "in vivo" towards the hyaline cartilage phenotype is addressed not only by the presence of growth factors but also by the biomechanical environment that the cells sense in the joint cartilage (Nugent-Derfus 2007). Thus, the success of a regenerative strategy depends to a high extent on the way in which the medium that surrounds the transplanted cells is able to transmit to them the same compression forces to which cells are subjected in a healthy joint cartilage. This is the role of the scaffold in cartilage tissue engineering; cells are seeded into a macroporous polymeric sponge which is placed in the site of the cartilage defect. The morphological and mechanical properties of the

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material in addition to the interaction between cells and pore surfaces, define to a large extent the biomechanical environment that the cells experience after being transplanted, and also the characteristics of the tissue they are able to produce and organize (Martínez-Díaz, 2009).

In this work we show a procedure to obtain a very versatile series of materials with varying mechanical and other physical properties, and show to what extent the mechanical properties of the scaffold determine the histological characteristics of the regenerated tissue in a rabbit knee model. This study is performed using biostable polymers; thus, there is no influence of degradation products in the non-vascularised cartilage tissue, nor of the loss of mechanical properties of the scaffold over time.

2 MATERIALS AND METHODS

2.1 Scaffold Preparation

In this work biostable polymeric scaffolds were prepared using a template technique. Poly(methyl methacrylate) microspheres with 90 microns diameter in average (PMMA Colacryl DP 300, Leucite International) were used as porogen. The microspheres were introduced into a mold consisting of two glass plates separated by a rubber ring. The mold was placed into a hot press at 170 ºC to allow PMMA microspheres to soften, and then compressed to form a template, approximately 2 mm thick, the interconnection points between porogen microspheres creates the pore throats in the scaffold, thus, pore interconnectivity is controlled by the pressure applied when producing the template.

Monomer mixtures of ethyl acrylate, EA (Aldrich, 99 %), and hydroxyethyl acrylate, HEA (Aldrich, 96 %) or methacrylic acid, MAAc (Aldrich, 99 %), were prepared adding different amounts of triethyleneglycol dimethacrylate, TrEGDMA (Aldrich 98% pure) as crosslinking agent and 1 % of 98 % pure benzoin (Scharlau) as photoinitiator. The templates were immersed in the monomeric solutions and polymerized under ultraviolet light at room temperature. After polymerization, the template was dissolved with acetone for approximately 48 h by means of a Soxhlet extractor. Then, scaffolds were immersed in a glass with a large excess of acetone and the solvent was changed slowly by water to allow the uniform contraction of the scaffolds. Replicas of the scaffolds were cut in 3 mm diameter and washed with water/ethanol for 6 hours in Soxhlet extractor. Finally, scaffolds were dried in vacuo for 24 h at room temperature and then another 24 h at 50 ºC.

The scaffold samples were sterilized with gamma radiation, at a dose of 25 kGy before implant.

The morphology of the scaffold was examined in a cryogenic scanning electron microscope (cryoSEM) (JEOL JSM 5410) equipped with a cryounit (Oxford CT 1500). The samples were mounted on copper stubs and gold coated using a sputter coater. The microscope was used with an acceleration tension of 15 kV.

2.2 Animals

Adult male New Zealand rabbits, weighing 1.5-2.0 kg were obtained from Granjas San Bernardo S.L. (Tulebra, Spain) and kept under conventional housing conditions. Quarantine lasted 7 days. Animals were housed with appropriate bedding and provided free access to drinking water and food. Rabbits were kept in standard single cages under controlled temperature and light conditions.

The study protocol was approved by the Ethics Committee of the Universidad de Valencia according to 86/609/EEC law and 214/1997 and decree 164/1998 of the Generalitat Valenciana.

2.3 Scaffold Implant

Rabbits were preanaesthetized by subcutaneous injection of 15 mg/kg Ketamine (Ketolar®, Pfizer laboratories) and intramuscular injection of 0.1 mg/kg Medetomina (Domtor®, Pfizer laboratories), and prepared before surgery (washed, shaved, etc.). Then, general anaesthesia was induced by 4% isofluorane using a specially designed mask and maintained by administration of 1.5% isofluorane with O_2 (2 l/min). The surgical site was sterilized using iodine solution and rabbit non-sterile parts were covered with sterile drapes. All surgeons wore sterile coats and gloves, and all instruments were sterilized and kept sterile during the operation. Scaffolds were moistened with phosphate buffered saline (PBS), and vacuum was applied to assure liquid penetration into the porous cavities before implanting. Two rabbits were used for each group.

An arthrotomy at the knee joint was performed through a medial longitudinal parapatellar incision. The medial capsule was incised and the patella laterally dislocated. A 3-mm steel trephine was used to create a chondral defect, 3 mm in diameter and 1 mm in depth, in the central articulating surface of the trochlear groove. The defect was cleaned and

rinsed with sterile saline, and scaffolds were laid into the defect and held in place by repositioning the patella within the trochlear groove. Arthrotomy and skin were sutured with continuous stitches of 4/0 Coated Vicryl® (Johnson-Johnson Intl) After removal of the conformed anaesthesia mask, all rabbits were returned to their cages and allowed free cage activity. Postoperative analgesia consisted of intramuscular injection of 3 mg/kg dexketoprofen (Enantyum®, Menarini laboratories) on the surgery day and the same dose every 24 h for 3 days. At the end of surgery, 3 mg/kg intramuscular injection of Gentamicine (Genta-Gobens®, Normon laboratories) was administered as antibiotic prophylaxis.

2.4 Animal Sacrifice and Tissue Retrieval

Three months after scaffold implant, rabbits were sacrificed with a lethal intravenous injection of anesthetic overdose in the auricular vein (500 mg/iv Tiopental; Tiobarbital®, Braun laboratories). A cut of 10x10x5 mm was made in the articulations with implanted scaffolds, and special care was taken in order to keep the repaired defect at the centre of the sample in order to asses cartilage repair by histological and immunohistological analyses.

2.5 Histological Studies

Morphology was studied following standard histological procedures. Briefly, rabbit articulation specimens were rinsed with PBS and fixed with 4% formaldehyde at room temperature for 5 days. Then, samples were rinsed with PBS and decalcified with Osteosoft decalcifier solution (Merck) during 5 weeks at room temperature. Finally, the specimens were embedded in paraffin, and 5 μm thick serial sections were obtained in order to localize the middle part of the scaffolds (with a diameter of approximately 3 mm), that were stained with Haematoxylin-Eosin and Masson's trichrome.

The ability of chondrocytes to synthesize glycosaminoglycan (GAG) within the porous scaffold was monitored by Alcian blue staining (pH 2.5), counterstained using Harris haematoxylin. Stained sections were analyzed under Leica optical microscope (Leica DM 4000B) and pictures were taken using a camera (Leica DFC 420).

2.6 Immunohistochemistry

Standard immunohistochemistry techniques were performed to detect the collagen type I and type II, osteocalcin and Ki-67 expression. Anti Ki-67 antibody (MIB-1, DakoCytomation, Glostrup, Denmark) at 1:50 dilution, incubated at RT during 60 min, was employed to detect proliferating cells. Section of human neuroblastoma was used as a positive control. A mouse anti-Collagen I antibody (Sigma, Madrid, Spain) at 1:200 dilution and mouse anti-Collagen II antibody (Calbiochem, Madrid, Spain) at 1:200 dilution, incubated at -4ºC overnight, were used to study the synthesis of Collagen type I and type II. Rabbit cartilage and bone areas surrounding the scaffold were used as positive control. Osteocalcin detection was performed using a mouse anti-osteocalcin antibody (R&D Systems, Abingdon, UK) at 1:100 dilution and incubated at -4ºC overnight. Rabbit subcondral bone was used as positive control. As a negative control for each specific staining, the preimmune serum was substituted for the primary antibody.

Sections were deparaffinized and rehydrated through graded ethanol, rinsed in distilled water and treated with 0.3% H_2O_2 and 10% normal horse serum to block endogenous peroxidase and nonspecific binding, respectively. Antigen retrieval for collagen type I, II and Ki-67 was performed by pressure cooker boiling for 3 minutes in 10 mmol/L of citrate buffer (pH 6.0). For osteocalcin detection, slides were permeabilized using 0.1% Triton X-100 for 5 min and antigen retrieval was performed using 0.5% trypsin for 30 min at 37°C in a humidified chamber. Envision amplification system Dako (Cytomation Envision+System labeled polymer-HRP anti-mouse) was used, followed by revelation with 3,3'-diaminobenzidine (DAB, Dako) as chromogen according to the manufacturer's instructions. Sections were finally counterstained with Mayer's hematoxylin (Sigma).

3 RESULTS AND DISCUSSION

3.1 Scaffold Morphology and Properties

The pore architecture of the scaffold is determined by the porogen template. As shown in Figure 1, the pores have spherical form with a diameter that is related to the size of the porogen microspheres, but depends on the expansion or contraction of the material during the template extraction and drying as

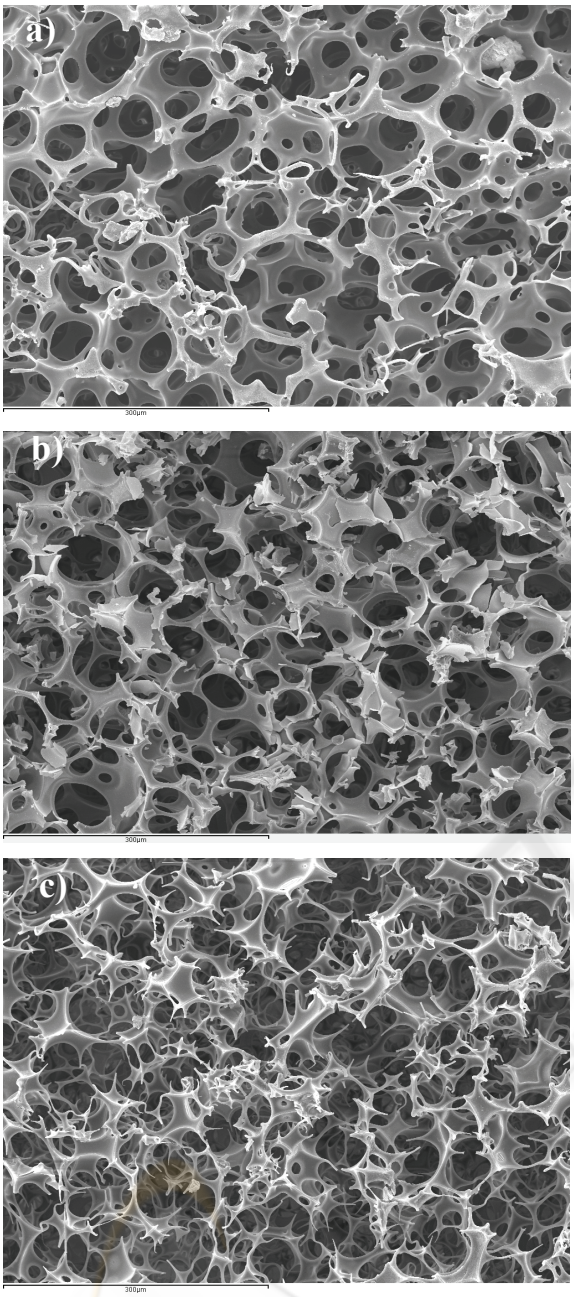

Figure 1: Scanning electron microscopy pictures of cross-sections of PEA scaffolds cross-linked with 5% (a), 10% (b) or 20 wt % (c) of TrEGDMA (see text).

well. We will further explain this point below. In this way a broad interval of pore sizes is available, since porogen microspheres made of different materials (polymeric particles, sugar microspheres, gelatine, paraffin and others) can be produced with diameters ranging from the micrometer to the millimetre scale (Brígido Diego 2005, Chen 2004, Ma 2003). The pore interconnection is determined by the compression of the porogen microspheres at high temperatures followed by cooling under pressure. Larger or smaller pore throats can thus be obtained. Figures 1 to 3 show three polymeric scaffolds made of coplymer networks in which one of the comonomers is ethyl acrylate and the other one is triethyleneglycol dimethacrylate, TrEGDMA, a tetrafunctional cross-linker.

The effective extraction of the porogen and any low molecular weight substance remaining in the material after the polymer synthesis is a requirement of any scaffold aimed as an implant or cell culture support. In our materials, the fact that the polymer is synthesized in the form of a copolymer network allows swelling it in a suitable solvent of the porogen, thus facilitating template extraction. Nevertheless, simple drying of the scaffold after this operation usually produces the collapse of the pore structure (Brígido Diego 2007). In the example of the scaffolds shown in Figure 1, when the PEA is polymerized inside the template with low cross-linking density and then immersed in acetone, it absorbs a large amount of solvent, swells significantly and the glass transition of the polymer decreases to very low temperatures. Thus, when the template completely disappears, the PEA network is swollen in acetone and consequently very soft, and in addition the pores are filled with acetone. If acetone is evaporated in those conditions, the scaffold collapses and the structure of the dry polymer becomes non porous. Slow exchange of the good solvent, acetone, by a bad solvent, water in this case, allows the controlled contraction of the sponge avoiding pore collapse. The properties of the material from which the scaffold is made: cross-linking density, solubility and glass transition temperature highly affect the tendency of the scaffold to close the pores (Brígido Diego 2005). Figure 1c shows how a highly cross-linked polymer network can be produced with an extremely porous structure (up to 95% volume fraction of pores) while maintaining mechanical consistency.

Physical and mechanical properties of the scaffold can be tailored by combining different monomers. For the animal model four series of materials were prepared: poly(ethyl acrylate), PEA, copolymers of ethyl acrylate and hydroxyethyl acrylate, P(EA-co-HEA), containing 10 or 50% by weight of HEA or copolymers of ethyl acrylate and methacrylic acid, P(EA-co-MAAc) containing 10% MAAc. Triethyleneglycol dimethacrylate at a ratio of 5 wt% with respect to the rest of monomers was used as cross-linking agent. PEA scaffold is hydrophobous, P(EA-co-MAAc) 90/10 and P(EA-

co-HEA) 90/10 are slightly hydrophilic, and so the bulk polymers are able to absorb 2.3 and 3.3% of water (measured on dry basis) when immersed in liquid water to equilibrium, and finally P(EA-co-HEA) 50/50 is a hydrogel whose equilibrium water content is 18.1% of water (Campillo-Fernandez 2007).

3.2 Animal Model

After implantation, scaffolds completely filled the 3 mm diameter chondral defect, with their external surface aligned with the articular surface of the trochlear grove. After sacrifice, macroscopic observation at the implant zone revealed good integration into the osteoarticular complex in all animals.

As an example of tissue remodelling three months after implantation, Figure 2 shows the cross section of the implant site stained with hematoxylin-eosin, where the material of the scaffold appears as the white regions in these pictures. Figure 2a shows that a layer of tissue has been formed on the surface of the scaffold. This tissue contains cells isolated in lacunae and ordered in columns, and histology and inmunostaining show that it contains type II collagen and glycosaminoglycans. The thickness of this layer was very variable in the different animals. In some cases a thin layer of hyaline cartilage was found (as that shown in Figure 2a), while in other cases the scaffold is much displaced in depth towards the trabecular bone, penetrating the layer of subchondral bone. On another hand, the stiffer scaffolds maintained their original shape with the pore structure filled with cartilaginous tissue showing expression of type II collagen and glycosaminoglycans, and with cells isolated in lacunae, as shown in the detail of Figure 2b at high magnification. Some cells are close to the pore walls, but most of them appear in the centre of the cavities. Interestingly enough, some of the cells were Ki67 positive, indicating that they had proliferating capacity. In the region of the scaffold touching the bone tissue some capillaries were apparent and positive staining for bone markers (osteocalcin and type I collagen) was found. On the contrary, P(EA-coHEA) 50/50 hydrogels suffered a high deformation which closed the pore structure, in which nearly no cells were found.

Since no cells were implanted with the scaffold, all tissue regeneration must be assumed to come from the messenchymal cells arriving to the site of the implant, coming with blood flowing during operation or from synovial fluids. Those cells placed on top of the scaffold are subjected to compression forces that lead to differentiation towards the hyaline cartilage phenotype.

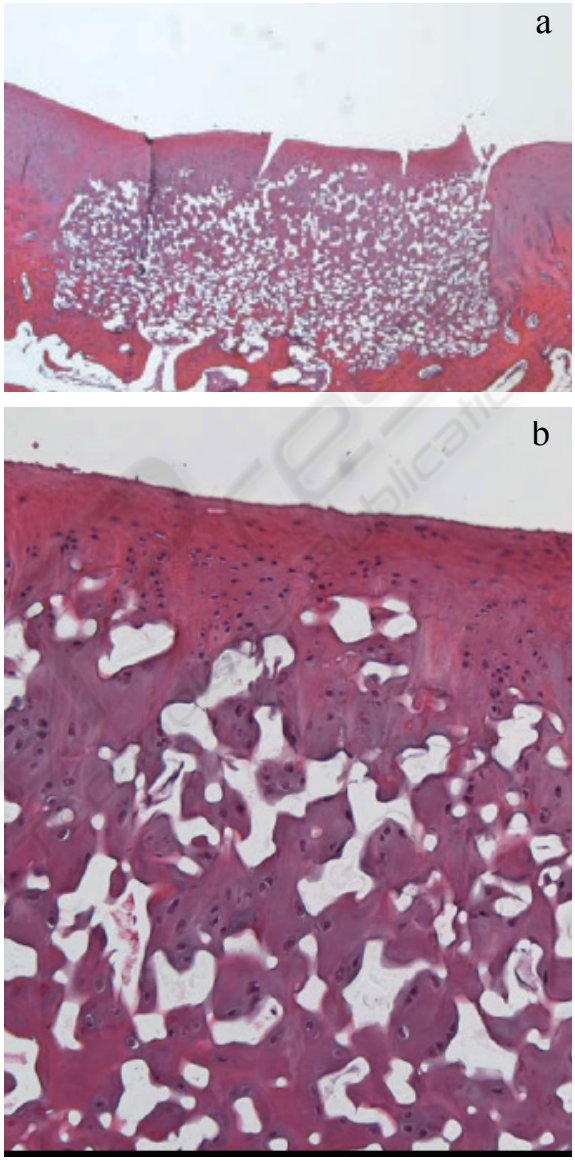

Figure 2: PEA scaffold integration in the surrounding tissue three months after implant, stained with haematoxylin-eosin.

These cells give rise to a layer of well differentiated hyaline cartilage on top of the scaffold, with chondrocytes isolated in lacunae ordered in columns perpendicular to the joint surface. In this way, the external surface of the regenerated tissue acquires the characteristics that resemble the original one. The growth of this tissue layer moves the scaffold towards subchondral bone, in a greater or lesser extent depending on the animal

and on the stiffness of the material. Other cells invade the pore structure of the scaffold and differentiate to chondrocytes as well, producing sufficient extracellular matrix to fill the pores.

4 CONCLUSIONS

A broad series of biostable polymer networks can be polymerized in the free spaces of a template made of sintered microspheres. The dissolution of the template allows producing the macroporous scaffold, whose pore structure depends on that of the template but also on the drying protocol and on the properties of the material, in particular its glass transition temperature and solubility in the solvents employed to extract the template. When scaffolds made with this procedure were implanted in a knee joint model, without any pre-seeded cells, good integration of the scaffold in the host cartilage tissue was observed in all animals after three months, with an even surface in the zone of the defect. Tissue regeneration comes from the differentiation of mesenchymal cells arriving to the site of the implant and invading the scaffold. These cells are able to produce neotissue with the characteristics of hyaline cartilage. This work shows the important role of the scaffold as the support that allows mechanical stresses to be transferred to cells during cartilage regeneration “in vivo”, and the possibility of regenerating joint cartilage without the artificial supply of autologous chondrocytes or pluripotential cells to the site of the implant.

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