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Ultrasound stimulus to enhance the bone regeneration capability of gelatin cryogels

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Abstract— **In the present study, gelatin-based cryogels have been seeded with human SAOS-2 osteoblasts. In order to overcome the drawbacks associated with *in vitro* culture systems, such as limited diffusion and inhomogeneous cell-matrix distribution, this work describes the application of ultrasounds (average power, 149 mW; frequency, 1.5 MHz) to physically enhance the cell culture *in vitro*. The results indicate that the physical stimulation of cell-seeded gelatin-based cryogels upregulates the bone matrix production.**

I. Introduction

A recurrent strategy in the field of Tissue Engineering is the development of porous scaffolds containing bioactive compounds (e.g. proteins) and aiming at the formation of new tissue *in vitro* or *in vivo* [1,2]. Formerly, numerous synthetic and natural biomaterials have been proposed as cell scaffolds: synthetic polymers include poly(caprolactone), poly(DL-lactic acid), poly(DL-glycolic acid) and derivatives thereof, whereas natural matrices include collagen and gelatin [3]. In this work, gelatin was selected, since it is a self-assembling, non-toxic, biodegradable, inexpensive and non-immunogenic biomaterial [4]. Gelatin has already been applied in medicine as a wound dressing [5] and gelatin-based sponges showed their potential in the field of Tissue Engineering [6]. Normally, gelatin is processed into a hydrogel at ambient temperature, but, interestingly, hydrogels can also be synthesized via a cryogenic treatment. Lozinsky *et al.* have referred to porous hydrogels obtained by cryogenic treatments using the name "cryogels" [7]. Our gelatin cryogels were prepared as scaffolds for human cells [8,9] and were characterized by well-defined "curtain-like" pore architecture [6].

To enhance the biomimetic properties of our gelatin-based cryogels, a physical stimulus (i.e. low-power ultrasounds) was applied on cell-seeded scaffolds. Previous research already indicated the potential of ultrasound stimulation on cell-seeded biomaterials such as titanium and hydroxyapatite [10,11]. Fassina *et al.* showed that the ultrasound stimulus caused the upregulation of cell proliferation and of extracellular matrix (ECM) deposition onto the biomaterial surface. As consequence, a natural bioactive coating (composed by cells and their ECM) was obtained avoiding the above-mentioned approach where the biomimetic properties of cell carriers are improved only by a protein coating applied before the cell seeding. In the present work, we combine polymeric scaffolds (i.e. gelatin-based cryogels) with ultrasound stimulation in order to introduce a natural biomimetic and bioactive surface coating.

II. Materials and Methods

A. Hydrogel synthesis and characterization

In the present work, a novel porous gelatin-based cryogel with a pore size of 135 ± 10 μm diameter was applied. Methacrylamide-modified gelatin type B was used as starting material. The gelatin applied was isolated from bovine skin by an alkaline process (Rousselot). The material possessed an approximate iso-electric point of 5 and Bloom strength of 257. The synthesis of methacrylamide-modified gelatin was performed as described earlier [12]. Part of the amine functions of gelatin were reacted with methacrylic anhydride. For this work, a derivative with a degree of substitution of 60%, based on the lysine and hydroxylysine units, was used [12]. In a subsequent step, the modified gelatin was used for the production of 10% (w/v) hydrogels [8]. Shortly, the hydrogels were obtained by dissolving 1 g gelatin type B, previ-

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ously modified with methacrylamide side groups, in 10 ml double distilled water at 40°C, containing 2 mol% photo-initiator Irgacure® 2959 (Ciba Specialty Chemicals N.V.), as calculated to the amount of methacrylamide side chains. The solution was then injected into the mould of a cryo-unit, after which the solution was allowed to gel for 1 h at room temperature. In a final step, the hydrogel was exposed to UV-light (276 nm, 10 mW/cm^2, Vilber Lourmat) for 2 h. Next, a cryogenic treatment was applied as described in detail in a previous paper [8]. The hydrogels were cooled from 21°C until -30°C at a cooling rate of -0.15°C/min. After incubating the sample for 1 h at the final freezing temperature, the frozen hydrogel was transferred to a freeze-dryer to remove the ice crystals, resulting in a porous scaffold. The hydrogels were sterilized using ethylene oxide (cold cycle, 37°C) prior to cell seeding. The visualization of the porous structure was performed using micro-computed tomography (micro-CT) analysis and scanning electron microscopy (SEM). For the micro-CT analysis, a "Skyscan 1072" X-ray micro-tomograph was used as described in detail previously [6]. Shortly, the system consisted of a X-ray shadow microscopic system and a computer with tomographic reconstruction software. The porous gelatin cryogel was scanned at a voltage of 130 kV and a current of 76 μA. For the SEM analysis, a Fei Quanta 200F (field emission gun) scanning electron microscope was used to image the gold-sputtered sample.

B. Cell seeding

The human osteosarcoma cell line SAOS-2 was obtained from the American Type Culture Collection (HTB85, ATCC). The cells were cultured in McCoy's 5A modified medium with L-glutamine and HEPES (Cambrex Bio Science), supplemented with 15% fetal bovine serum, 2% sodium pyruvate, 1% antibiotics, 10^{-8} M dexamethasone, and 10 mM β-glycerophosphate (Sigma-Aldrich). Ascorbic acid, another osteogenic supplement, is a component of McCoy's 5A modified medium. The cells were cultured at 37°C in the presence of 5% CO_2. The gelatin-based cryogels were placed inside two culture systems: the "static", that is, the control well-plate without external stimulus and the "ultrasonic", that is, the ultrasonically stimulated well-plate. A cell suspension of 4×10^5 cells in 100 μl was seeded on the top of each cryogel and, after 0.5 h, 1 ml of culture medium was added to submerge the scaffolds with medium. The cells were allowed to attach overnight, while in a subsequent step, the control culture continued without ultrasound stimulus, whereas the ultrasound culture was stimulated 20 min/day.

C. Ultrasound stimulus

An ultrasound stimulus [10,11] was applied through the culture medium by a FAST ultrasound generator (Igea, Carpi, Italy) to the seeded gelatin cryogels. The mechanical wave had the following characteristics: signal frequency equal to 1.5 ± 0.03 MHz, duty cycle of 200 ± 4 μs, repetition rate equal to 1 ± 0.02 kHz, and average power of 149 ± 3 mW. The ultrasound culture was placed into a standard cell culture incubator with an environment of 37°C and 5% CO_2, and it was stimulated 20 min/day for a total of 22 days. The culture medium was changed on days 4, 7, 10, 13, 16, and 19.

D. Control culture

The control culture was placed into a standard cell culture incubator. The duration of the control culture was 22 days and the culture medium was changed on days 4, 7, 10, 13, 16, and 19.

E. Determination of DNA content

The cells were lysed by a freeze-thaw method in sterile deionized distilled water and the released DNA content was evaluated using a fluorometric method (PicoGreen, Molecular Probes). A standard DNA curve, obtained from a known amount of osteoblasts, was applied in order to express the results as cell number per scaffold [13].

F. Extraction of the bone matrix

At the end of the culture period, the cultured scaffolds were washed with sterile PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH=7.4) four times for 10 min in order to remove the culture medium, and then incubated for 24 h at 37°C with 1 ml of sterile sample buffer (1.5 M Tris-HCl, 60% [w/v] sucrose, 0.8% [w/v] Na-dodecyl-sulphate, pH=8.0). At the end of the incubation period, the sample buffer aliquots were removed and the total protein concentration in the two culture systems was evaluated by the BCA Protein Assay Kit (Pierce Biotechnology). The calibration curves to measure the extracted proteins were performed by an ELISA assay [14,15]. The results are expressed as fg/(cell×scaffold).

G. Statistics

Results are expressed as mean ± standard deviation. In order to compare the results between the two culture systems, one-way Analysis of Variance (ANOVA) with *post hoc* Bonferroni test was applied, electing a significance level of 0.05.

III. Results

A. Hydrogel development and characterization

The porous biomaterial functioning as a cell support was prepared by a cryogenic treatment of crosslinked methacrylamide-modified gelatin. First, the modified gelatin was synthesized by reacting part of the lysine and hydroxylysine units of gelatin type B with methacrylic anhydride as described in detail in a previous paper [12]. The degree of substitution of the gelatin used in the present study was 60% (determination via ^{1}H-NMR spectroscopy), based on the gelatin primary amine functions. Next, hydrogels were formed by gelation of an aqueous methacrylamide-modified gelatin solution, followed by radical crosslinking using a UV-active photo-initiator. Finally, the chemically crosslinked hydrogels were subjected to a cryogenic treatment. The pore size, morphology, and porosity of the 3D scaffold developed were analysed by micro-CT and SEM analysis. The pore size as studied by SEM analysis (Fig. 1A), was in the range of 135 ± 10 μm. Interestingly, in addition to a non-destructive visualization of the scaffold architecture (Fig. 1B), micro-CT analysis also enables to determine the porosity distribution throughout the entire scaffold. Fig. 2 indicates

that the porosity of the gelatin-based cryogel developed ranges from 77% up to 84%.

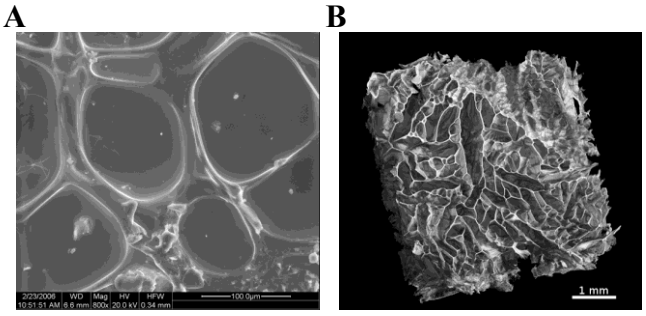

Figure 1. SEM image (*A*) and micro-CT image (*B*) showing the pore size and morphology of the gelatin-based cryogels developed.

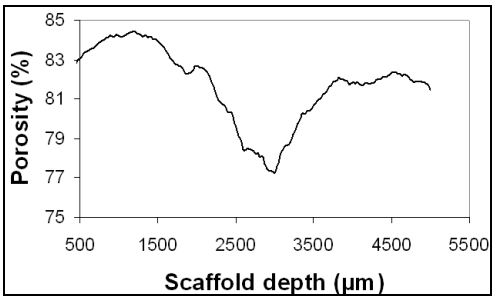

Figure 2. Scaffold porosity as a function of the depth obtained via micro-CT analysis.

In a subsequent part of the present work, the gelatin-based cryogels were evaluated for their potential to support the proliferation of SAOS-2 osteoblasts and their extracellular matrix production under static condition and ultrasound stimulus. Therefore, the human SAOS-2 osteoblasts were seeded onto the surface of the gelatin-based cryogels, followed by culturing them without or with an ultrasound stimulus for 22 days. These culture methods enabled to study the SAOS-2 cells as they modified the biomaterial surface through cell proliferation and through the production of extracellular matrix deposited onto the gelatin-based cryogels. The cell-matrix distribution was compared between the two culture systems.

B. Cell proliferation

For the static culture, the cell number per disk increased to $21.4\times10^5 \pm 5.7\times10^5$, while, for the ultrasound culture, the cell number was $23.1\times10^5 \pm 4.5\times10^5$ ($p>0.05$).

C. Extracellular matrix extraction

In order to evaluate the amount of bone extracellular matrix secreted onto the gelatin cryogels, an ELISA of the extracted matrix was performed. The results indicated that the ultrasound stimulation significantly increased the production and the deposition of various extracellular matrix proteins onto the surface of the gelatin biomaterial ($p<0.05$) (Table I).

IV. DISCUSSION

In order to improve the biomimetic properties of gelatin-based cryogels, an ultrasound stimulus was applied after cell seeding. The results indicated that the ultrasounds did not affect the cell proliferation. However, the physical stimulus did result in an upregulation of the production of some extracellular matrix proteins such as type-I collagen, osteopontin, and osteocalcin (Table I). Interestingly, the above-mentioned proteins are fundamental constituents of the physiological bone matrix. In particular, type-I collagen is the most important and abundant structural protein of the bone matrix. Osteopontin is an extracellular glycosylated bone phosphoprotein secreted at the early stages of the osteogenesis before the onset of the mineralization; the protein binds calcium and is likely to be involved in the regulation of the hydroxyapatite crystal growth; moreover, it promotes cell attachment through specific interaction with the vitronectin receptor. Osteocalcin is secreted after the onset of mineralization and binds to bone minerals.

TABLE I. EXTRACELLULAR MATRIX CONSTITUENTS ONTO GELATIN DISKS.

	Total ECM production in fg/(cell×scaffold)		
	Control (C)	Ultrasound (U)	U/C
Osteocalcin	1.23 ± 0.12	3.60 ± 0.27	2.93-fold*
Osteopontin	6.12 ± 0.53	14.80 ± 0.45	2.42-fold*
Type-I collagen	29.00 ± 6.21	44.05 ± 4.51	1.52-fold*

Table note: * $p<0.05$.

The obtained results can be clarified using Pavalko's signaling model [16]. The ultrasound mechanical stimulation brings about both an increase of the Ca^{2+} flux into the osteoblast cytosol as well as the release of the intracellular Ca^{2+}. According to the model, the increase of the cytosolic Ca^{2+} concentration is the starting point of signaling pathways targeting specific bone matrix genes.

The use of a cell line already indicated the potential of the ultrasound stimulation in combination with the gelatin-based cryogels developed. Interestingly, we anticipate that, upon fine-tuning the parameters of the physical stimulation, autologous bone marrow stromal cells could also be applied instead of SAOS-2 osteoblasts in order to realize full immunocompatibility with the treated patient. In the present work, a gelatin-based cryogel was combined with an ultrasound stimulation in order to develop tissue-engineering constructs enabling bone repair. The results clearly indicated that the physical stimulation resulted in the upregulation of extracellular matrix proteins.

In this study, elaborating an idea of Castner and Ratner [17], we physically enhanced the coating of gelatin with osteoblasts and with extracellular matrix: we followed a particular biomimetic strategy where the seeded cells built a new biocompatible surface over the biomaterial, making it very useful for the biointegration. The idea of Castner and Ratner and a discussion about the concept of biocompatibility follow. When a biomaterial is implanted in a biological environment, a nonspecific and nonphysiologic layer of adsorbed proteins mediates the interaction of the surrounding host cells with the material surface. The body interprets this protein layer as a foreign invader that must be walled off in an avascular and tough collagen bag. Therefore, the biomedical surfaces must be developed so that the host tissue can recognize them as "self". Castner and Ratner think the biocompatible surfaces of the biomaterials that heal as the surfaces with the characters of a "clean, fresh wound": these "self-

surfaces" could obtain a physiological inflammatory reaction around the biomaterials leading to normal healing, leading to physiological osteointegration in bone tissue engineering.

In the present study, we followed a particular biomimetic strategy: we obtained a surface coating of the biomaterial, over which the seeded and physically stimulated osteoblasts built a new biocompatible surface made of cell-matrix layers, that is, a physiological surface with the characters of a "clean, fresh wound". Using the preceding biomimetic tissue-engineering approach [10,14,15,18-28], gelatin-based cryogels could be combined with differentiated cells and their extracellular matrix proteins as implants for bone repair in clinical applications.

V. Conclusion

In conclusion, we theorize that the obtained cultured self-surface could be used fresh, that is, rich in autologous cells and matrix, or after sterilization with ethylene oxide, that is, rich only in autologous matrix. In future work, we intend to use our constructs, which are rich in autologous matrix, as a simple, storable, tissue-engineering product for bone repair.

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