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Development of a Packed Bed Bioreactor for Cartilage Tissue Engineering

Efficient transport of regulatory molecules is one of the main requirements for directing the growth and differentiation of bone marrow stromal cells (BMSC). We have designed and utilized a packed bed bioreactor system for cultivation of murine BMSC immobilized in alginate microbeads produced by electrostatic droplet generation. Continuous medium perfusion at velocities that are physiological for cartilage and bone (~ 100 μm/s) provided convective mass transport through the packed bed while the small bead diameter (~ 500 μm) ensured short diffusion distances to the immobilized cells. Over 5 weeks of cultivation, the cells remained viable at a constant density whereas the alginate microbeads retained size and spherical shape. Cell density used in this study (5×10^6 cells/ml) was found to be too low to result in cartilage tissue formation. However, in several cases, loosely bonded groups of beads and merged beads without visible boundaries were observed, implying that higher cell densities may lead to development of a continuous extracellular matrix. This study indicates the potentials of the packed bed bioreactor system in conjunction with alginate microbeads as cell carriers, for precise regulation of the cellular microenvironment in cartilage tissue engineering.

Keywords: cartilage, alginate microbeads, packed bed bioreactor, tissue engineering

1. INTRODUCTION

Articular hyaline cartilage is an avascular tissue containing only one cell type, the chondrocyte, which generates and maintains the extracellular matrix (EMC) consisting of a fibrous network of collagen type II and glycosaminoglycan (GAG) – rich proteoglycans. The main function of articular cartilage is to allow joint mobility while transferring compressive and shear forces. Hyaline cartilage has a very limited capacity for intrinsic functional repair. Small chondral defects have been reported to regenerate by cell migration but untreated, large, full – thickness defects heal with formation of fibrocartilaginous repair tissue. This tissue significantly differs in composition from the normal cartilage resulting in inferior biomechanical function and progression to osteoarthritis with an eventual need for a total knee replacement surgery [1].

Surgical and biological attempts have been made to induce significant and durable repair response in cartilage defects. The main objectives are symptom relief and the re–establishment of normal load – bearing function of the articular surface. Current therapies for cartilage replacement include allografts and artificial plastic and metal implants, which suffer from problems such as lack of available donor tissue, rejection,

increased susceptibility to infection and toxicity. Other treatment techniques focus on attempts to recruit progenitor cells from the bone marrow by penetration of the subchondral bone or transplantation of osteochondral grafts, periosteum or perichondrium. However, all of these therapies promote formation of a fibrous repair tissue showing acceptable short–term results but long–term outcomes are usually unfavorable [2, 3].

Tissue engineering is one of the new strategies that have emerged over the past decade to address the clinical problem of tissue failure [4, 5, 6]. It is particularly significant because it can provide biological substitutes of compromised native tissues. Also, engineered tissues can serve as physiologically relevant models for controlled studies of cells and tissues under normal and pathological conditions [7, 8]. Tissue engineering combines cells with biomaterials, synthetic or natural, and environmental factors to induce differentiation signals into surgically transplantable formats and promote tissue repair and/or functional restoration [6, 7]. Despite many advances, researchers in this field still face significant challenges in repairing, replacing or regenerating specific tissues or organs.

Cartilage tissue engineering based on the integrated use of chondrogenic cells, biodegradable scaffolds, and bioreactors was proposed for *in vitro* regeneration of tissue equivalents that would ideally have site- and scale-specific structural and biomechanical properties of native articular cartilage, and integrate firmly and completely to the adjacent host tissues [6, 7].

Cells used in this approach are chondrocytes, fully differentiated cells, or osteochondral progenitor cells isolated from bone marrow (herein referred to as bone marrow stromal cells – BMSC). When selecting the optimal cell type to use, one must consider the proliferative capacity of the cells *ex vivo*, phenotypic stability, and immunogenicity. The proliferative capacity of fully differentiated cartilage adult cells is limited and long – term *in vitro* expansion can reduce their functional quality. The other limitation is the amount of tissue that can be harvested as well as donor site morbidity. On the other hand, BMSC are potential cell source with the excellent proliferative and regenerative capacity. However, compared to cultures of differentiated chondrocytes these cells require additional biochemical regulatory signals to induce chondrogenic differentiation. The process of stem cell chondrogenesis is directed by a number of growth factors and signaling molecules in addition to cell – cell, and cell – matrix interactions and biomechanical factors. Efficient and controlled delivery of biochemical signals is thus one of the main requirements in cultures of BMSCs. Today the challenge still lies in designing a microenvironment to provide the correct instructions to bring those cells to the desired functional state to create structurally organized cartilage [8, 9].

Biomaterials in different forms are used to provide three – dimensional support for cells to reside, proliferate and produce matrix. The cell support has to be porous to allow for effective transport of nutrients and waste products as well as biocompatible with the host tissue. It should be also biodegradable by hydrolytic or enzymatic mechanisms, leaving non-toxic degradation products that can be eliminated from the body. In addition, the biomaterial support must have the appropriate physical and mechanical properties for the given application, so to withstand physiologic loading until sufficient tissue is regenerated [8, 9].

Hydrogels are a class of biomaterials that have been used widely in the tissue engineering and drug delivery fields. A hydrogel is composed of highly cross–linked hydrophilic polymer chains to yield a highly swollen, water insoluble gel structure. Such environment has been shown to support chondrocyte survival and cartilage matrix synthesis [9]. The hydrophilicity of hydrogels prevents protein adsorption thereby minimizing foreign body responses when implanted *in vivo*; but this property also reduces the ability of the hydrogel to exert a biological influence onto anchorage dependent cells [9] Alginate is one of the most important scaffold materials since when crosslinked with calcium ions, it forms a gel that is biocompatible, biodegradable, and it is easily processed into different shapes. When immobilized in alginate matrix chondrocytes were shown to retain their phenotype and produce cartilaginous components [9, 10, 11] In addition, bone marrow cells were shown to proliferate and express chondrogenic differentiation when entrapped and cultivated in highly pure alginates [13].

Bioreactors for cartilage tissue engineering should maintain controlled conditions in culture medium (e.g., temperature, pH, osmolality, levels of oxygen, nutrients, metabolites, regulatory molecules), facilitate mass transfer, and provide physiologically relevant physical signals (e.g., interstitial fluid flow, shear, pressure, compression) [7]. Interstitial fluid flow was particularly proposed for enhancement of mass transfer directly to the cells so that cartridges with porous scaffolds perfused by the medium were used for tissue engineering of myocardium [14, 15], cartilage [16, 17, 18] and bone [19, 20].

The goal of this study was to develop a bioreactor system with interstitial medium flow coupled with alginate as a cell support for cartilage tissue engineering. We have previously shown that murine BMSC could be effectively immobilized in alginate microbeads produced by electrostatic droplet generation [21, 22]. In the present work, we have developed a packed bed bioreactor for cultivation of BMSC immobilized in alginate microbeads under continuous perfusion of the culture medium.

2. MATERIALS AND METHODS

2.1 Cell isolation and expansion

Inbred CBA mice, 8 – 10 weeks old, were used as donors of bone marrow cells, which were propagated *in vitro* [22]. Pooled femoral bone marrow cell suspensions were prepared in Dulbecco.s Modified Eagle Medium (DMEM), centrifuged for 10 min at 1200 rpm and re – suspended in expansion medium, *i.e.* DMEM supplemented with 10% Fetal Bovine Serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 0, 1 mM non-essential amino acids, - all from Sigma (St. Luis, MO) and 1 ng/ml bFGF (human recombinant, R&D Systems, Minneapolis, MN). Cells were plated in 25 cm^2 culture flasks at 2 - 4 x 10^6 cells/ml in 6 ml of expansion medium and cultured in a humidified 5% CO_2 incubator at 37°C. After 3 days, 3 ml of expansion medium was added to each flask and thereafter every 3 days the medium was completely replaced. BMSCs were selected based on their ability to adhere to the tissue culture plastic and at about 80 % confluence (approximately after 7 – 10 days of culture) the cells were detached by using 0.25% trypsin/1 mM EDTA and then re–plated at 16 – 26 x 10^3 cells/cm^2. Second passage BMSCs were cultured in the same manner, and harvested at about 80 – 90% confluence (approximately 11 days).

2.2 Production of alginate microbeads with immobilized cells

Sodium alginate powder (Sigma, St. Luis, MO) was dissolved in WFI water at a concentration of 2.2 % w/w and then mixed with a suspension of BMSCs in culture medium to obtain final concentrations of 1.5 %w/w alginate and 5 x 10^6 cells/ml. Alginate microbeads were produced by electrostatic droplet generation [21,22]. In brief, cell/alginate suspension was extruded through a positively charged blunt stainless steel needle (24 gauge, 6,4 kV applied potential) at a constant flow rate of 14,0 ml/h by a syringe pump and resulting droplets were collected in a gelling bath (1.5% w/v $CaCl_2$), as shown in the Figure 1. As Na ions were exchanged with

Ca ions, alginate droplets hardened and formed insoluble microbeads with entrapped cells. The microbeads were left for 30 min in the gelling bath in order to complete gellation and then were transferred into the bioreactor.
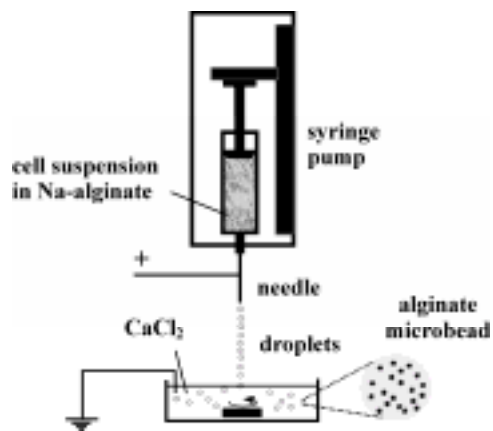

Figure 1. Experimental set-up for electrostatic generation of alginate microbeads with entrapped BMSC

2.3 Bioreactor cultivation

The packed bed bioreactor was custom made at the Faculty of Technology and Metallurgy, Belgrade. It consisted of two glass halves, which were fit into a piece of silicone tubing to form a chamber of 1 ml in volume (Figure 2). At the ends of glass halves were sintered glass plates, which provided retainment of the microbeads in the chamber. The silicone tubing enabled continuous gas exchange.
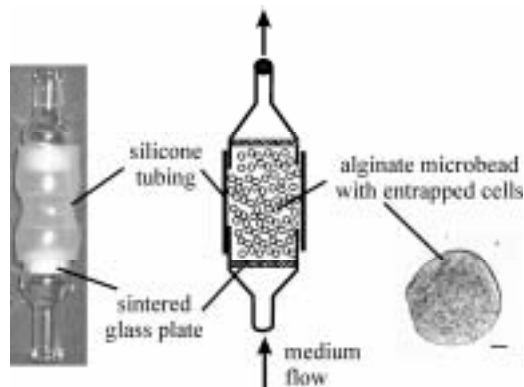

Figure 2. Packed bed bioreactor: two glass halves with sintered glass plates at the ends were fit into a piece of silicone tubing to form a chamber for cultivation of alginate microbeads (scale bar 100 μm)

The packed bed bioreactor was connected to a peristaltic pump and a medium reservoir using silicone tubing that ensured gas exchange in the culture medium (Figure 3). Two syringes were used for medium exchange and debubbling. Bioreactor system was placed in a humidified 5% CO_2 incubator at 37°C.

Alginate microbeads (1 ml; 5×10^6 cells/ml) were placed in the bioreactor chamber and the system was filled with 12 ml of the culture medium. Medium for cultivation was DMEM supplemented with 10% Fetal Bovine Serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml vitamin C, 10 nM dexa-

methasone, and 10 ng/ml TGF-β1 (R&D Systems, Minneapolis, MN). Perfusion rate in the system was set to 0.18 ml/min. During the 5 weeks of cultivation, 40% of medium was exchanged twice a week.
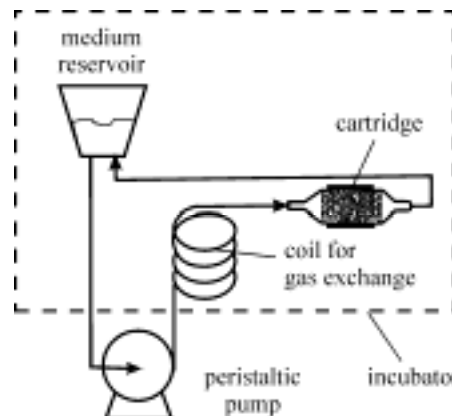

Figure 3. Bioreactor system: the packed bed bioreactor is connected to a recirculation loop consisting of a medium reservoir and silicone tubing coild for gas exchange.

2.4 Alginate microbead characterization

Diameters of microbeads were measured with an accuracy of 10 μm using an optical microscope. The average microbead diameter and standard deviations were then calculated from the measured data of 20 – 30 microbeads. Cell concentrations and viabilities were determined following dissolution of microbeads in 0.05M Na –citrate (1g of microbeads in 5 ml of Na-citrate solution). BMSC cell concentration was estimated by using a Thoma counting chamber and cell viability was assessed by means of trypan blue staining technique.

Alginate microbeads sampled for histology were fixed in 2.5 % glutaraldehyde, embedded in araldite, cross-sectioned (1.5 μm) by LKB III Ultramicrotom and stained with safranin-O and toluidine blue.

3. RESULTS

Electrostatic extrusion resulted in spherical alginate microbeads of about 500 μm in diameter with cell concentration of 5×10^6 cells/ml. Over 5 weeks of cultivation in the packed bed bioreactor, the microbeads maintained consistency and spherical shape. Immobilized cells remained viable at approximately constant concentration showing no significant proliferation during the cultivation period. Most of the alginate microbeads remained separated and only occasionally loosely bonded groups of microbeads were observed (Figure 4). These bonds were probably formed by secretion of cells at the bead surfaces, noticed in several cases (Figure 5). Some of the microbeads appeared void of cells (Figure 4) probably due to a lower initial cell concentration in these beads. In very rare cases, merged microbeads without visible boundaries between them were observed (Figure 6).

Histological staining has shown groups of viable cells resembling chondrocytes in lacunae (Figure 7). Safranin – O stained non – specific to alginate matrix.
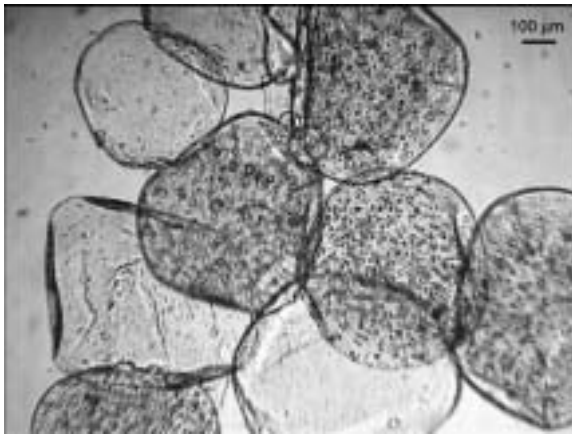
Figure 4. Loosely bonded group of microbeads. Sporadic beads void of cells could be observed.
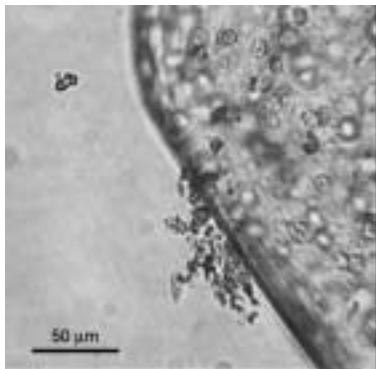

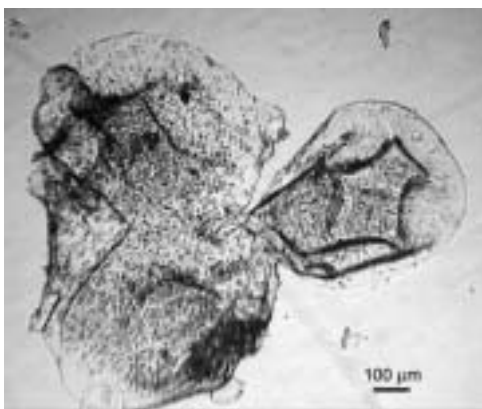**Figure 5. Cells at the microbead surface**

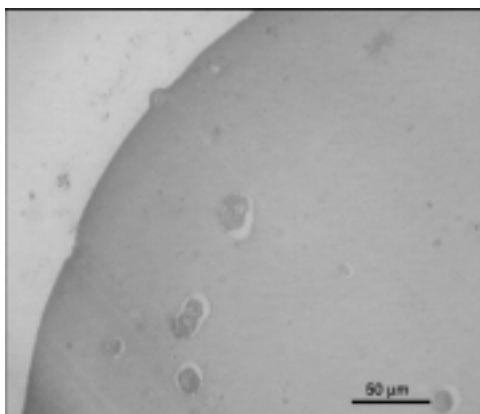**Figue 6. In very rare cases merged microbeads were observed**

Figure 7. Histological section of an alginate microbead cultured for 5 weeks in the packed bed bioreactor (toluidine blue staining)

4. DISCUSSION

Efficient mass transfer of regulatory molecules is one of the most important requirements in cultures of BMSC in order to induce desired lineage of differentiation. Bioreactors with medium perfusion through porous scaffolds seeded with cells were proposed for creation of convective mass transport through the cultivated tissue. However, in this bioreactor design, very low medium velocities are required since the cells are directly exposed to the fluid flow. Packed bed bioreactor design with cells entrapped in alginate particles, on the other hand, can be beneficial for providing protection of cells from fluid flow and shear stresses. However, this bioreactor design imposes a requirement to use small diameter alginate particles (< 1mm) in order to provide short diffusion distances. In this study, we have investigated potential application of a packed bed bioreactor with alginate microbeads as support for BMSC for cartilage tissue engineering.

Alginate microbeads were produced by the electrostatic droplet generation technique, which was shown previously to provide controlled production of microbeads down to 50 µm in diameter [23,24]. Electrostatic extrusion of BMSC suspension had little effect on the cell viability and BMSC entrapped in alginate microbeads remained viable for 4 weeks in a static culture [22]. In the present study, we have cultivated alginate microbeads in a packed bed bioreactor with medium perfusion, which provided more efficient mass transfer to the beads. Perfusion rate was about 100 µm/s, which corresponded to the range of blood velocities found in capillaries. In this experimental set-up, the cells would ideally regenerate cartilaginous matrix leaving the channels mimicking a capillary network. In the present study, bonds between alginate microbeads and merged microbeads were observed only sporadically, which was probably due to a generally low initial concentration of cells used. Cell density was shown to strongly affect the rate of ECM deposition in alginate immobilized chondrocytes such that wet weight fractions of GAG and collagen were more that 3 fold higher in constructs with initial cell density of 64×10^6 cell/ml as compared to the constructs initially seeded with 4×10^6 cell/ml [12]. In addition, microbeads void of cells observed in the present study correspond to findings that biodegradable fibrous scaffolds seeded with chondrocytes at low densities collapsed after 12 days in culture and confirm the hypothesis that there is a minimal cell concentration required for cell survival and ECM synthesis [25]. However, merged microbeads observed occasionally in the present study, imply that higher cell concentrations could result in formation of a continuous ECM integrating neighboring beads.

Finally, the system based on alginate microbeads and the packed bed bioreactor could have an additional potential for cartilage tissue engineering by incorporation of encapsulation and controlled delivery of regulatory molecules in the vicinity of the immobilized BMSC. Alginate matrix itself could be used to retain specific biologically active molecules [26] or it could incorporate a second delivery system

following the approach used in hydrogel systems with immobilized chondrocytes and microparticles with encapsulated TGF-β1, which were shown to stimulate ECM deposition under in vitro conditions [27,28]. Interstitial medium flow and controlled delivery of regulatory molecules in such system could provide precise regulation of the cellular microenvironment needed for BMSC cultures.

5. CONCLUSION

In this study, we have utilized a packed bed bioreactor system with continuous perfusion of the culture medium for in vitro cultivation of murine BMSC immobilized in alginate microbeads produced by electrostatic droplet generation. Perfusion rate was set to physiological blood velocities while the small bead diameter ($\sim$ 500 μm) provided short diffusion distances. Over 5 weeks of cultivation, cells remained viable at a constant cell density in preserved alginate microbeads, which were sporadically found to form loosely bonded groups. Only in very rare cases, merged microbeads without visible boundaries were found. However, the obtained results imply that the cell density used in this study was too low and that higher cell densities may lead to formation of a continuous ECM in this system. The present work indicates potentials of the packed bed bioreactor system in conjunction with alginate microbeads for efficient delivery of regulatory molecules to the immobilized cells and precise control of the cellular microenvironment in cartilage tissue engineering.

ACKNOWLEDGEMENTS

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РАЗВОЈ БИОРЕАКТОРСКОГ СИСТЕМА СА ПАКОВАНИМ СЛОЈЕМ ЗА ПРИМЕНУ У ИНЖЕЊЕРСТВУ ТКИВА ХРСКАВИЦЕ

Андреа Осмокровић, Бојана Обрадовић, Диана Бугарски, Бранко Бугарски, Гордана Вуњак-Новаковић

Ефикасан пренос регулаторних молекула је један од основних захтева у културама ћелија костне сржи ради подстицаја ћелија на диференцијацију у жељеном правцу. У овом раду је развијен и примењен биореакторски систем са пакованим слојем за култивацију ћелија костне сржи миша имобилисаних у алгинатне микрочестице произведене техником електростатичке екструзије. Континуалним протоком медијума при физиолошким брзинама струјања (~ 100 μm/s) обезбеђен је конвективни пренос масе у пакованом слоју док су малим пречником честица (~ 500 μm) остварена мала растојања за дифузиони пренос масе до имобилисаних ћелија. У току 5 недеља култивације, ћелије су остале вијабилне при константној ћелијској густини док су алгинатне микрочестице задржале величину и сферичан облик. Показало се да је концентрација ћелија коришћена у овом раду (5 x 10^6 ћел/ml) сувише ниска за формирање ткива хрскавице. Међутим, у неколико случајева нађене су слабо везане групе микрочестица, а понегде и потпуно стопљене микрочестица без приметних граница што наводи на претпоставку да би при вишим концентрацијама ћелија био могућ развој континуалног екстрацелуларног матрикса. Резултати добијени у овом раду указују на могућност примене биореакторског система са пакованим слојем алгинатних микрочестица, носача ћелија, за прецизну регулацију микро-околине ћелија у инжењерству ткива хрскавице.