Biomimicry of PAM Microfabricated Hydrogel Scaffold

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Abstract

The most important issue in fabricated scaffolds in tissue engineering is to reproduce both the mechanical and chemical ECM properties, in order to promote cells adhesion and proliferation. In particular a specific issue seems to be important in cells reproduction, is the environment in which cells are seeded. In order to realize an artificial construct able to mimic the EMC properties, many materials have been studied and used in years, and recently the attention has undergone to hydrogel. The reason on hydrogel's fame is due to their viscoelastic characteristics, biocompatibility, easiness of fabrication in different shapes, and quickly mass transfer between nutrients and cells.

In this paper we have optimized the working conditions in order to realize a well defined hydrogel scaffold, with a specific shape and tuning mechanical properties using a novel microfabrication technique developed and patented in our laboratory, PAM system. Moreover the microfabricated Three-Dimensional (3D) alginate scaffold was designed and characterized in order to include cells.

Introduction

The development of biomaterials for tissue engineering applications has recently focused on the design of biomimetic materials that are capable to reproduce the Extra Cellular Matrix (ECM) properties[1-3]. The biomimetic materials, such as biopolymers[4, 5]] and hydrogels[6, 7], potentially mimic many roles of ECM in tissues. For example, biomimetic scaffolds can provide biological cues for cell–matrix interactions to promote tissue growth and differentiation. Realize scaffolds that reproduce both chemical and mechanical ECM characteristic is an important issue for engineering tissues. Scaffolds are well known as replacing tissue substitutes, restoring the structure and function of the native tissue. The scaffolds provide the basic 3D topology[8], and mechanical framework[9] for the cells to attach and proliferate before they can differentiate into a tissue. These 3D matrices guide tissue growth in a directed and controlled way. The feasibility to include cells into scaffolds could promote a new way of interaction, allowing to cells the possibility to a spatial self-organization. The cell viability, growth, behavior and tissue formation are influenced not only by the scaffold design and its microenvironment, but also by its biochemical and biophysical properties[10, 11]. Hydrogels in tissue engineering meet a number of design criteria to function appropriately and promote new tissue formation, such as classical physical parameters (degradation and mechanical properties) as well as biological performance parameters (cell adhesion, proliferation and metabolism). An absolutely critical parameter is the biocompatibility of hydrogels, that relates to the ability of material to exist within the body without damaging adjacent cells or lead to undesired response (such as the inflammatory response).

Nowadays several microfabrication techniques are well known for the realization of synthetic polymeric scaffold. In particular Rapid Prototyping techniques, based on CAD/CAM system, have been developed, such as 3D printing [12-13], laser sintering [14], fused deposition modeling [15-16], multi-phase jet solidification [17], bioplotter[19], indirect solid-free form fabrication[20], or Pressure Activated Microsiringe (PAM)[21], [22]. After a first feasibility study of these Rapid Prototyping techniques, evaluating limits and problem due to the hydrogels processing[23], we propose a new microfabrication protocol purposely tuned for the realization of hydrogel scaffolds. We designed a new rapid prototyping microfabrication system, PAM2 system[24], for the extrusion of relatively high viscous material. The system provide a controlled CAD/CAM motion system, and a controlled extrusion system. In order to realized well-defined structure we characterized not only the extrusion method, but also the realized structure. PAM2 system could realize simple structures, as well as more complex shape such as rectangular, hexagon, and octagonal cell unit grid, and so on, using a friendly user interface. Starting from these results it is possible to realize a desired scaffold, not only with defined dimensions and shape, but also with desired mechanical properties. Moreover we performed cellular test, in order to realize a new concept of scaffold that foresee the inclusion of cells. HepG2 cells were chosen to be included in alginate scaffolds, due to their well- known use to be encapsulated[25-27], and to its relatively high sensitivity to shear stress[28-29]. A proper cross-linking protocol was then chosen in order to reproduce liver mechanical properties[30], and hydrogel scaffolds including cells were microfabricated with PAM2 system. Cells vitality was evaluated, demonstrating that the extrusion method could not damage cells or induce any undesired cellular process, such as apoptosis.

Materials and Methods

Powder Alginic Acid Sodium Salt from Brown Algae (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in Phosphate Buffered Solution (PBS, Sigma-Aldrich, St. Louis, MO, USA) at different concentration, respectively at 4%, 6% and 8% (w/v), in order to reproduce sterile operative conditions necessary for cells environment and treatment. Alginate solution in PBS was sterilized at a temperature of 120°C for 30 minutes in autoclave.

The alginate microfabricated scaffolds were crosslinked with 1 ml of 0.1M Calcium Chloride (Sigma-Aldrich, St. Louis, MO, USA) solution in PBS for 1 minute.

Microfabrication of hydrogels scaffold

A feasibility study of microfabrication of hydrogel scaffolds was carried out. In order to realize defined microstructures, with a fixed topology, and known dimensions, a CAD/CAM system was set-up for the extrusion of viscous solution. In a preliminary analysis we decide to use the CAD/CAM moving system of an existing Pressure Assisted Microsyringe[20, 30] (PAM) system. This micro-positioner is able to move linearly in the plane, with a resolution and accuracy of 0.1μm, varying the moving velocity from 0 to 4.5 mm/s. Using a CAD/CAM user interface it was possible to draw the trajectories necessary to move the system, and micro-fabricate an hydrogel scaffold with a specific topology. Due to the relatively high viscosity values of alginate solutions, it was necessary to design an extrusion system capable to exert a constant force. A new system was developed and foresee the introduction of a new motor, with the aim to control the extrusion of viscous solutions. This new patented system is known as PAM2[23]. The extrusion process is controlled by a stepper motor (RS 440-420, RS Components S.p.A., Italy), chosen in order to exert a constant working torque. Using a lead screw mechanism it was possible to convert a rotational motion into a linear one. A screw of 2mm pitch was mounted on the motor, obtaining a resolution of 0.1 μm of linear motion. The stepper motor was able to exert a working torque of 0.07 Nm, and extrude homogeneously viscous alginate solution. Angular velocity of the stepper motor was chosen as the controller parameter of the system, in order to monitor the extrusion velocity of viscous solution.

A mechanical support was properly designed to allocate a 5mL syringe with a controlled stepper motor. A needle of 29 gauges was used to extrude alginate solution with lower width (width of 300 μm for the deposition line). Because of the necessity to characterize the working condition of the new microfabrication system, we firstly characterize the extrusion phase. The flow of viscous solution was monitored, in order to characterize the outflow solution velocity. Then, chose the extrusion set-up, we implemented to the CAD/CAM simple and linear trajectories in order to measure the deposed line width. A serpentine trajectory was implemented to the CAD/CAM system varying the motion velocity from 2.5mm/s to 4.5mm/s. The deposition line width was analyzed under optical microscope (Olympus AX70, Olympus Italia, Segrate, Italy), measuring each line in three different points and averaging the measurements. So the extrusion protocol used to realize defined hydrogel scaffolds was set-up.

Cell Test

HepG2 (human hepatocellular liver carcinoma cell line, ECACC, Porton Down, UK) were cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% (w/v) Fetal Bovine Serum (FBS, Sigma-Aldrich, St. Louis, MO, USA), 2mM of L-glutammine (Sigma-Aldrich, St. Louis, MO, USA), and 100 μg/mL of streptomycin (Sigma-Aldrich, St. Louis, MO, USA), and 100U/mL of penicillin (Sigma-Aldrich, St. Louis, MO, USA), and maintained in an incubator at 5% CO2 and 37°C[24]. In order to evaluate the influence of different alginate concentrations on cell viability[31], metabolic tests were performed using a cell suspension of HepG2 in alginate solutions. Powder Alginic Acid Sodium Salt from Brown Algae (Sigma-Aldrich, St. Louis, MO, USA) was solved in Phophate Buffered Solution (PBS) with concentration of 4%, 6%, and 8% (w/v), and autoclaved. Cells suspension was obtained gently mixing 10 million cells in 1 ml of 4%, 6%, or 8% of alginate solutions, in order to have an uniform cell distribution. Medium samples were collected at different time (6, 24, and 48 hours), used to evaluate glucose and urea concentrations. Glucose was assayed with Megazyme D-Glucose Assay Kit (Megazyme International Ireland Ltd., Bray, Ireland), 0.01mL of medium sample were added to 0.3mL of GOPOD (Glucose Oxidase/Peroxidase) Reagent and incubate at 45°C for 20 minutes, after this time absorbance was read at 510nm with the FLUOstar Omega microplate spectrophotometer (BMG LabTech GmbH, Offemburg, Germany). Urea was measured using a commercial kit (Sigma-Aldrich, St. Louis, MO, USA), 0.01mL of medium sample were added to 0.025mL of Urease Enzyme and incubated for 10 minutes at 37°C. After, 0.05mL of Phenol Nitroprusside Solution, 0.05mL of Alkaline Hypochloride Solution, and 0.2mL of deionized water were added and placed at room temperature for 20 minutes, and absorbance was read at 660nm.

Cell Extrusion System

Using the previously described PAM2 Rapid Prototyping system, high concentrate suspension of cells in alginate solutions were deposed in order to obtain hydrogel scaffolds with a well defined shape. Powder Alginic Acid Sodium Salt from Brown Algae (Sigma-Aldrich, St. Louis, MO, USA) was solved in PBS, and the sterilized in autoclave. 10 million HepG2 cells were gently mixed in 1mL of alginate solution, and then insert in a commercial sterilize syringe. System control parameters were set-up to proper values, and a specific shape was design in order to realize well defined hydrogel scaffolds including HepG2 cells. In particular, this cell line was chosen for a specific reason: hepatic cells exhibit a high sensitivity to shear stress induced on their membrane[28, 32]. The evaluation of the imposed shear stress was previously modelled and evaluated with the Software Comsol Multiphysics 3.2. By these results it was possible to set-up an extrusion protocol, realizing the desired hydrogel scaffolds.

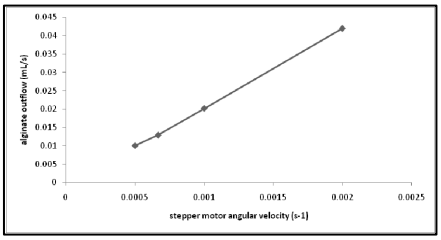
Results and discussions

Characterization of the new microfabrication system

In order to compare hydrogel scaffold realized with the two system, PAM and PAM2, specific tests were done in order to quantify typical features of a scaffold. As described by the extrusion model[20], lined width is strictly dependent with the motion system set-up. In particular it is possible to reduce the extruded line width increasing the relative velocity between the needle and the deposition plane. The design of the new microfabrication system PAM2 provides the inclusion of an extrusion motor to an existing CAD/CAM motion system. It simplifies the previously mentioned extrusion model, without any significant changes. Because the stepper motor provides a fixed working torque during the extrusion, motor's angular velocity was choosen as control parameter of the extrusion phase. A first

characterization evaluate the outflow of the viscous solutions as function of the stepper motor velocity. Supposing a typical Poiseuille flow through the syringe, it is possible to estimate the extrusion velocity of the material. As shown in figure1 both outflow and extrusion velocity increase with the stepper motor velocity. In reason of these consideration a proper microfabrication protocol was defined for the extrusion of high viscous solution for PAM2 system.

(a)



(b)

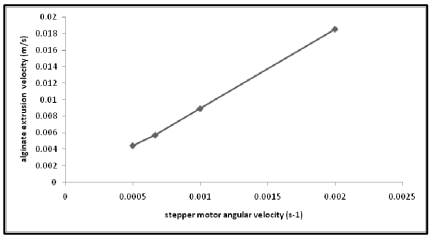
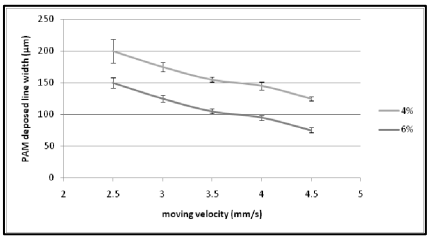


Figure 1. *Characterization of PAM2: (a) Outflow in function of stepper velocity, (b) Extrusion Velocity in function of stepper velocity*

After that, a characterization of the width of the deposed lines was done both for PAM and PAM2 microfabrication systems. In particular we decided to analyse change of line width as a function of the CAD/CAM stepper motor velocity. As expected by alginate solutions viscosity measurements, it was possible to realize structures of alginate solution at 4% and 6% (w/v) with PAM system. Instead of the realization of defined structure with higher viscosity value, such as 8%, with the PAM2 system. In particular, for PAM2, extruded line width characterization was performed starting from value of the outflow characterization. Stepper motor was set up for the maximum value of extrusion, imposed by the rheological tests, evaluating the worst case of deposition. Three serpentine two-dimensional patterns with stepper velocity respectively of 2.5, 3.5, and 4.5 mm/s were realized. As shown in figure 2, it is possible to evaluate a decrease of the deposited line width as function of the increase of motion velocity, both for PAM and PAM2. Using these results we set-up the extrusion parameters and the motion velocity in order to obtain a medium deposition line width of 120 μm for PAM, and width of 150 μm for PAM2. After this first part of characterization, we start to realize structure with more complex geometry such as rectangular and hexagon grids.

(a)



(b)

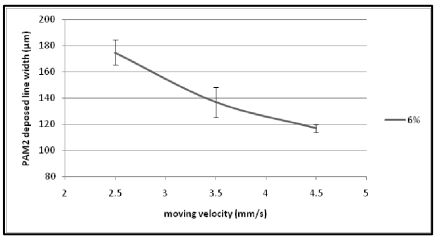
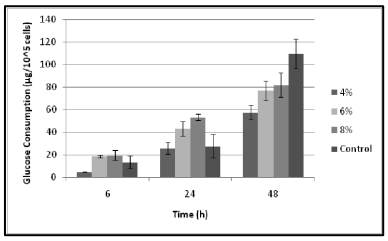


Figure 2. *Extruded line width with PAM(a); and with PAM2 (b)*

Cell Viability Test

In order to evaluate the influence of alginate concentration on cells viability, metabolic tests were performed on HepG2 cells. Measurements of glucose and urea on medium samples at different time (respectively 6, 24, and 48 hours) exhibit a trend similar to control, showing that alginate concentration does not influence significantly both glucose consumption and urea production. In particular glucose consumption shows a constant increase in all samples, with relatively higher values in alginate solutions of 6% and 8% as shown in figure 3a. At the same time, urea production exhibits a lower and constant increase, with respect to control without significant differences between the three alginate solutions (figure 3b).

(a)



(b)

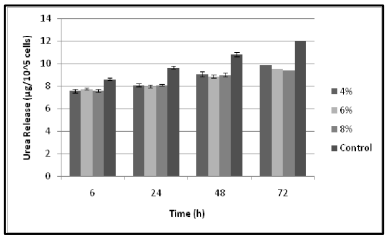


Figure 3. *Glucose consumption (a) and urea production (b) in static culture.*

Hydrogel scaffolds including cells

Starting from the characterization of the rapid prototyping techniques PAM and PAM2 during alginate solutions extrusion for the realization of well-defined geometries, and analyzed results shown by the extrusion model solved with Comsol Multiphysics, an extrusion protocol for hydrogel scaffolds was set up. In particular our attention was focused not only to the realization of microstructures with controlled dimension and mechanical properties, due to the fabrication parameter and the cross-linking reaction. In particular we chose to analyze a proper extrusion protocol for the microfabrication of scaffolds including cells. For this reason the PAM2 system was chosen because of the needle diameter, proper for cell typical dimension. Because of the results obtained with the rheological measurements, a minimum angular velocity was set up for the stepper extrusion motor. In this way it is possible to prevent eventually typical problems of viscosity increment during the extrusion, exhibited by alginate solutions. Motion velocity was set up to higher value, such as 4.5 mm/s. With this parameter set up it was possible to realize well defined alginate scaffold, with typical lines dimensions of 150 μm-250 μm. In order to obtain an homogeneous spatial distribution, rectangular grid distanced by 500 μm were realized with the CAD software and then converted to CAM commands. In order to reproduce mechanical properties of the hepatic tissue[29-39],, a 6% (w/v) alginate solution in PBS with an homogeneous and high concentrate distribution of HepG2 was used in the extrusion phase. Realised hydrogel scaffolds were then cross-linked with a 0.1M CaCl2 solution for 1 minute of reaction, and then rinsed with DMEM culture media in order to stop the cross-linking reaction. Cells viability test were then performed after 6 and 24 hours from the extrusion. With respect to cells control glucose consumption is a bit higher in samples of extruded cells, but significantly coherent with values obtained in previous tests. Instead, as expected, urea production is lower in extruded samples than in control. Typical values are not so different from the static control, as shown in figure 4. Because of this decrement it is possible to affirm that after extrusion there is a soft damage, HepG2 extruded cells tend to exhibit an increase of the metabolic/catabolic system during the period between 6 and 24 hours.

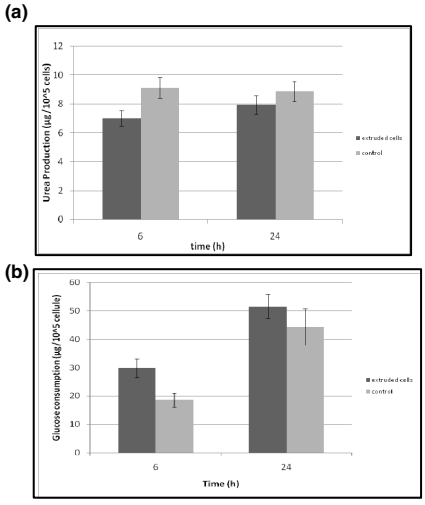


Figure 4 *Glucose consumption (a) and urea production (b) for extruded HepG2 cells.*

Results

In this study an innovative microfabrication method for the realization of biomimicry hydrogel scaffolds including cells is presented. After a feasibility studies of microfabrication techniques and materials properties, we concerned our attention on the realization of well defined alginate structures with two microfabrication systems, PAM and PAM2. A characterization of the microfabrication systems were performed, evaluating its advantages and limits. PAM2 system was chosen for the realization of alginate scaffold with embedded cells. A FEM analysis was performed in order to evaluate cell damage during the extrusion phase, due to the shear stress, choosing the proper microfabrication protocol. Alginate scaffolds including HepG2 cells were then realized, and their metabolic activity was analyzed with respect to control. Results shown that after a relative short time in which glucose consumption and urea production is lower to control, extruded cells exhibit a behavior similar to control.

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