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Design of novel 3D gene activated PEG scaffolds with ordered pore structure

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Abstract The ability to genetically modify cells seeded inside synthetic hydrogel scaffolds offers a suitable approach to induce and control tissue repair and regeneration guiding cell fate. In fact the transfected cells can act as local in vivo bioreactor, secreting plasmid encoded proteins that augment tissue regeneration processes. We have realized a DNA bioactivated high porous poly(ethylene glycol) (PEG) matrix by polyethyleneimine (PEI)/DNA complexes adsorption. As the design of the microarchitectural features of a scaffold also contributes to promote and influence cell fate, we appropriately designed the inner structure of gene activated PEG hydrogels by gelatine microparticles templating. Microarchitectural properties of the scaffold were analysed by scanning electron microscopy. 3D cell migration and transfection were monitored through time-lapse videomicroscopy and confocal laser scanning microscopy.

1 Introduction

Tissue engineering aims to promote the healing of diseased or injured tissues trough the use of a scaffold that supports cellular infiltration, contains bioactive signals, and is able to guide invading cells through tissue formation [1, 2]. The success of any tissue engineering approach mainly relies on the delicate and dynamic interplay among extracellular matrix (ECM) proteins, cytokines, growth factors, cell–cell contacts and mechanical stimuli that, when sapiently integrated and orchestrated within the scaffold, results in tissue or organ formation [3]. Therefore, biomaterial scaffolds have to provide mechanical and structural, as well as biological signals able to guide and direct cell functions [4]. In detail the scaffold-assisted regeneration of specific tissues has been shown to be strongly dependent on scaffold's surface/volume ratio, as well as on pore size and interconnectivity [5]. Indeed, these microarchitectural features significantly influence cell morphology, binding and phenotypic expression, as well as extent and nature of nutrient diffusion and tissue ingrowth [6–9]. It has also been suggested that the pore dimension may directly affect some biological events and, consequently different tissues require optimal pore size for their regeneration [6–8]. Therefore, scaffolds with significantly different micron-scale porosities are needed for regeneration of highly structured biological tissues. Furthermore, soluble macromolecules (e.g. growth factors, chemokines, cytokines) or insoluble factors (e.g. ECM proteins, glycosaminoglycans, and proteoglycans) have to be absorbed or covalently bound to scaffolds to allow them to provide biological signals. Although peptides and growth factors are generally used as the bioactive signals in tissue engineering, the employment of DNA is an alternative or complementary approach to introduce bioactive signals into scaffolds [10–15]. This approach provides the potential for long-term bio-availability of bio-active signals as cells themselves produce the proteins needed for the regenerative process. Scaffolds realised following this approach, were developed few decades ago and called Gene Activated Matrices (GAMs) [16–19]. They have been later implemented and successfully used in the field of bone, cartilage and skin tissue engineering [20–22]. More recently these scaffolds have been employed to implement the methodology of incorporation and release of nucleic acid within the matrix through formation of DNA complexes with cationic

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polymers [23] or by encapsulating plasmid in nanoparticle release systems [24].

In this article we present and discuss the results of a study aimed at preparing DNA bioactive poly(ethylene glycol) (PEG) porous hydrogel scaffold for tissue engineering. PEG hydrogels with finely controlled porous architectures were prepared via gelatine particles templating and then functionalised by poly(ethylene imine) (PEI)/DNA complexes adsorption. Furthermore, by controlling the gelatine microparticles spatial size distribution, the particle templating technique [25] was implemented in order to create and tailor porosity and pore size gradients within the porous architecture of the hydrogels. The ability of templated bioactive hydrogels to support cell attachment and migration through the interconnected structure, was evaluated, using fluorescently marked cells, by confocal laser microscopy and time-lapse videomicroscopy, respectively. Moreover the efficiency of these novel gene activated hydrogels was detected in terms of cell transfection (expression of green fluorescent protein (GFP)) in relation to DNA availability in the extracellular microenvironment.

2 Materials and methods

2.1 Gelatine microparticles preparation

Gelatine type B (Sigma–Aldrich, Mw = 176 KDa) with an isoelectric point (IEP) of 5.0, was used for microparticle fabrication. In particular, 5 g of gelatine were dissolved in 45 ml ddH_2O by mixing and heating (60°C). This aqueous gelatine solution was added dropwise to 250 ml of oil (Cotton Seed Oil Sigma–Aldrich) while stirring at 500 rpm. The temperature of the emulsion was then lowered to around 15°C with constant stirring. After 30 min, 100 ml of chilled acetone (4°C) was added to the emulsion. After 1 h, the resulting microparticles were collected by filtration, washed with acetone to remove residual oil, and mechanically sieved for size separation.

2.2 Hydrogels preparation

To generate porous hydrogels, we introduced 50% (v/v) of home-made uncrosslinked gelatine microparticles of specific diameter size into steel gaskets adhered to a glass slide. Then we poured a PBS solution containing 20–40% (w/v) of PEG diacrylate (PEGDA) (Sigma–Aldrich, Mw = 700 Da) and 3% of a UV light-sensitive radical (Irgacure 2959 Ciba, Switzerland) around the microparticles. This mixture was exposed to long-wavelength ultraviolet (UV) light (365 nm, 10 mW/cm^2) for 5 min in order to polymerize the diacrylate. After polymerization, the gelatine beads were leached away from the hydrogels using water at 37°C over 24 h. To obtain hydrogels with different pore size, templating particles of variable diameter size range (53–75; 75–150; 150–210; 210–300; 300–500 μm) were used. Moreover, two types of pore size distribution structure were realised: one with a stepwise porosity size gradient, characterised by two areas, each with a specific pore size, and another with a continuous gradient. In the first case, a partition was inserted in the middle of the gasket and then each of the two areas were filled with 75–150 and 300–500 μm diameter microparticles. In the second case, the gasket was filled with microparticles sized 75–150, 150–212, and 212–300 μm from bottom upwards.

2.3 Microstructural analysis

Hydrogel morphologies were investigated by Scanning Electron Microscopy (SEM) and image (imageJ®) analyses. Samples were serially dehydrated (50, 75, 85, 95% ethanol at 30 min each; 100% overnight), cross-sectioned, gold-sputtered, and analysed by SEM (S440, LEICA) at an accelerating voltage of 20 kV, and variable magnifications. The porosity was analysed in terms of pore size, shape and spatial distribution. In particular the mean pore diameter and the normalized pore size distribution were estimated by 2D image analysis procedures, tracing not less than 100 pores for each sample and correcting the software value, calculated with the hypothesis of spherical shape, with the factor $4/\pi$, according to the ASTM D3576 [26, 27].

2.4 Protein residual quantification

To verify if the radical-based polymerization mechanism leads to non specific covalent gelatine incorporation, a BCA protein assay (Micro BCA™ Pierce, Rockford, Illinois) on the final hydrogels was performed according to manufacture procedure.

2.5 Cell seeding and culture

NIH3T3 mouse embryo fibroblasts were cultured at 37°C and 5% CO_2 in DMEM (Invitrogen, Gaithersburg, MD) supplemented with 10% (v/v) FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin (HyClone, UK), in a humidified atmosphere at 37°C and 5% CO_2. Scaffolds for cell-culture experiments (d = 15 mm and h = 2 mm) were pre-treated by incubation in DMEM supplemented with 10% (v/v) FBS for 24 h. Before seeding, NIH3T3 cells were stained with green Cell Traker (Molecular Probes) according to manufacturer's procedures, in order to improve cell detection within scaffolds, and then trypsinized, harvested and centrifuged. 10^5 cells, resuspended in 200 μl of medium, were statically seeded onto samples representative of scaffolds with different pore dimension. After seeding, the

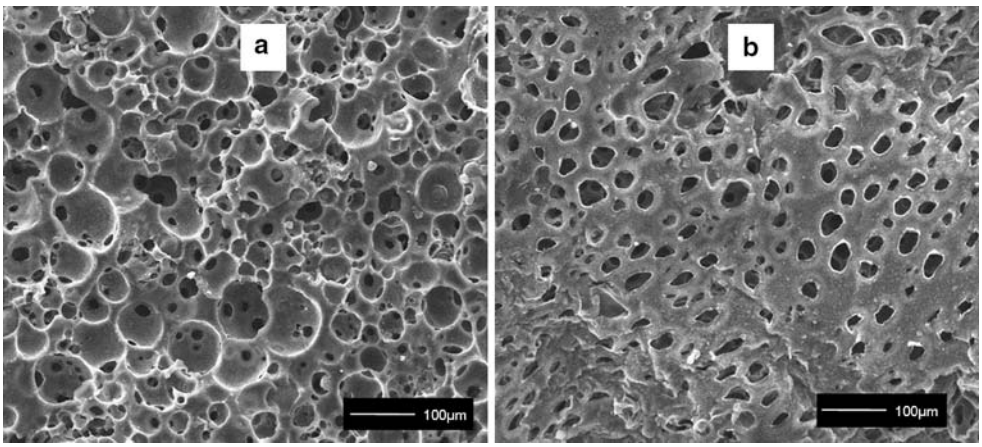

Fig. 1 SEM micrographs of **a** cross section and **b** surface of PEG hydrogel obtained using templating particles in the size range 75–150 μm

scaffolds were incubated for 2 h in a humified atmosphere (37°C, 5% CO_2), and subsequently, 1.5 ml of cell-culture medium was added to each sample. The samples were analysed by confocal laser scanning microscope (CLSM) (LSM510, Zeiss) after 24, 48 and 72 h of culture to investigate cell adhesion and penetration. Images were acquired by using a 20× objective, HeNe laser ($\lambda = 543$ nm) and z-stack function. Qualitative analyses of cell movement into the scaffold were also performed by time-lapse videomicroscopy using a fluorescence microscope and 10× objective, in order to evaluate cell behaviour in 3D porous structure. Images were acquired every 10 min over 6 h. Long term viability of cells in hydrogels from 24 h to 22 days was analyzed as a function of pore dimension by Alamar Blu assay (Invitrogen). 10^5 cells were statically seeded onto different samples (templating gelatine microparticles diameter of 53–75, 75–150, 150–300 300–500 μm). Experiments were repeated in triplicate for each pore dimension.

2.6 Complexes formation

Plasmid DNA encoding for green fluorescent protein (GFP), purified from bacteria culture using Qiagen extraction kit (Santa Clara, CA), was complexed with PEI (Linear PEI 7 mM amine content, Polyplus-transfection, Illkirch, France) at a nitrogen/phosphate ratio (N/P) of 5. Both plasmid DNA and PEI were diluted with NaCl (150 mM) and than mixed by adding PEI solution to DNA solution.

2.7 3D cell transfection

Scaffolds preparation and cell seeding for cell-transfection experiments were performed using the previously described procedures for preparing hydrogels with uniform pore size distribution (same dimension gelatine microparticles), but incubating the scaffolds with 200 μl of complexes (N/P = 5) solution for 24 h in order to induce complexes adsorption, and using unstained cells (without cell-tracker). Before cells seeding, complexes solution was completely removed and then the scaffolds were washed to remove non-adsorbed complexes. Retained complexes were quantified as a function of the pore size via a standard curve, by

Table 1 Templated PEG hydrogel pore size

Templating particles diameter (μm)	Dehydrated pore mean diameter (μm)
53–75	40 ± 9
75–150	81 ± 17
150–210	112 ± 16
210–300	171 ± 20
300–500	277 ± 26

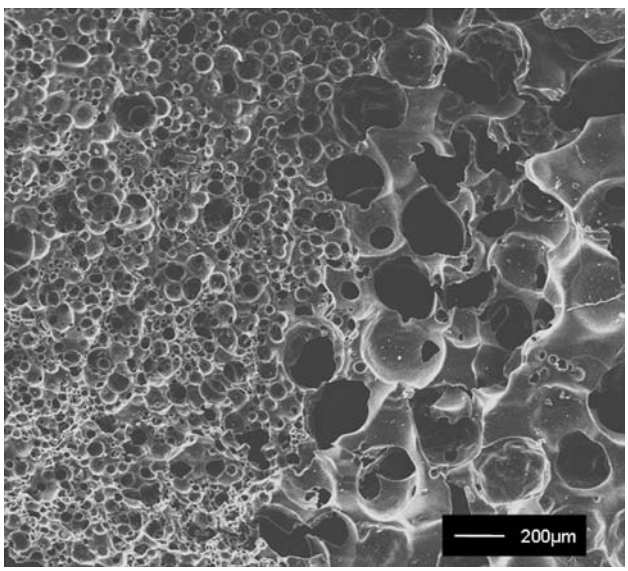

Fig. 2 SEM micrograph of stepwise pore size gradient hydrogel prepared with 75–150 and 300–500 μm templating particles

measuring the fluorescence at 535 nm of the washed water in a multi-well plate spectrofluorimeter (Perkin-Elmer, Wallac 1412). For this analysis, DNA was complexed with fluorescein-conjugated linear PEI, JetPEI-fluoF (Polylus-transfection, Illkirch, France). The detected values were used to determine the complexes retaining efficiency, that was expressed as the percent difference between the amount of total and non absorbed complexes. Experiments were repeated in triplicate for each pore dimension.

The efficiency of gene transfer by the DNA activated matrix was detected through fluorescence microscopy. Samples were investigated by CLSM at 24, 48, 72 and 96 h of culture in order to detect the distribution of GFP expressing cells.

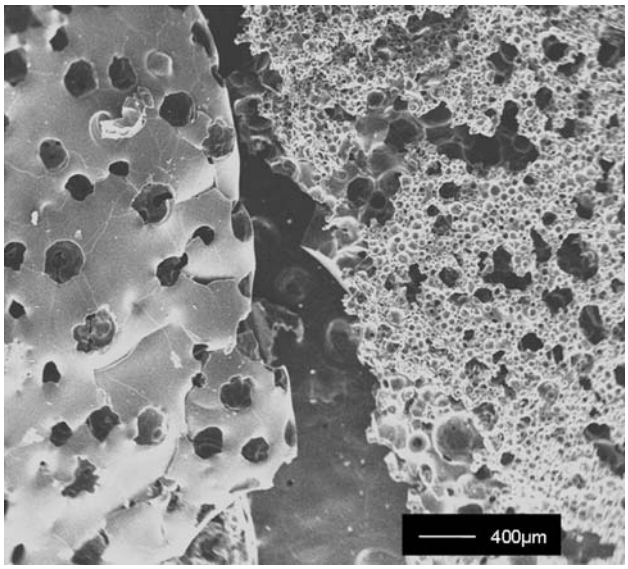

Fig. 3 SEM micrograph of front and bottom surfaces of a continuous pore size gradient hydrogel prepared using microparticles sized 75–150, 150–212, 212–300 μm from bottom upwards

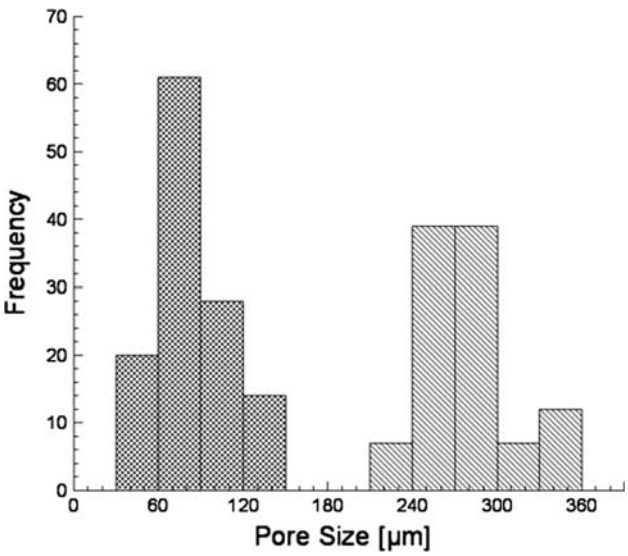

Fig. 4 Pore-size frequency distribution of stepwise gradient PEG hydrogel

3 Results and discussion

Assessment of the fundamental relationships among gene delivery, structural scaffold features, and tissue formation,

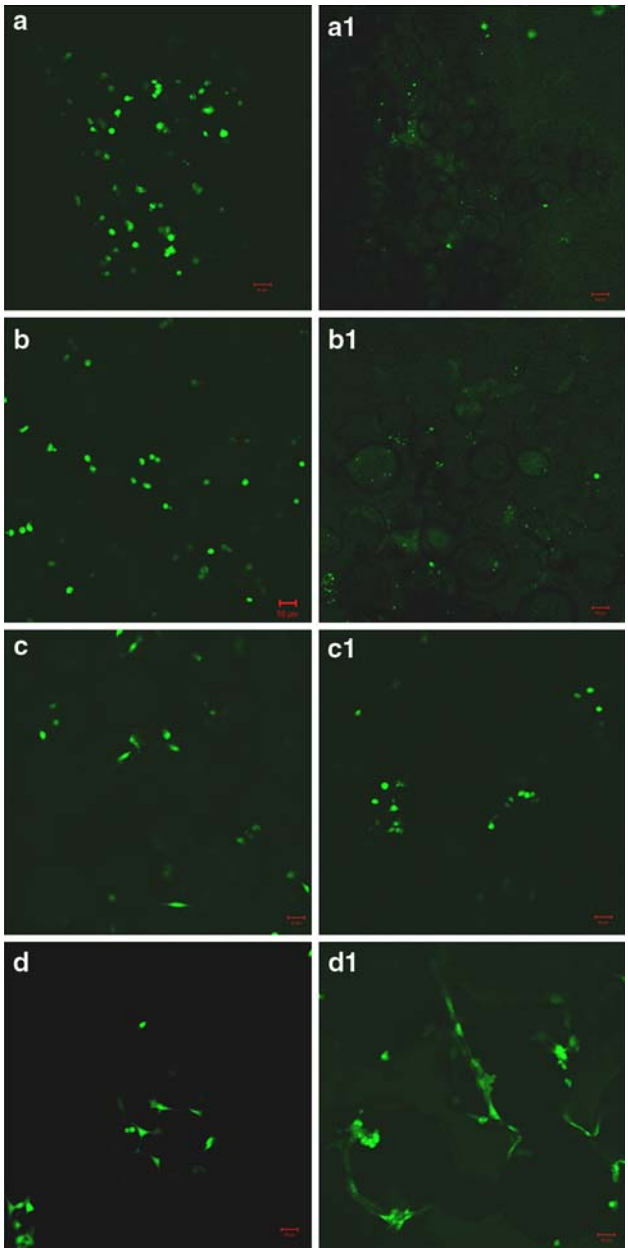

Fig. 5 CLSM images of cells within 3D matrices after 72 h of culture. Images a through d refer to the cell seeding surface, while a1 through d1 to the opposite surface. From *top* to *bottom* the image couples are related to matrices obtained using as templating agent gelatine microparticle with diameter of 53–75, 75–150, 150–300 and 300–500 μm, respectively

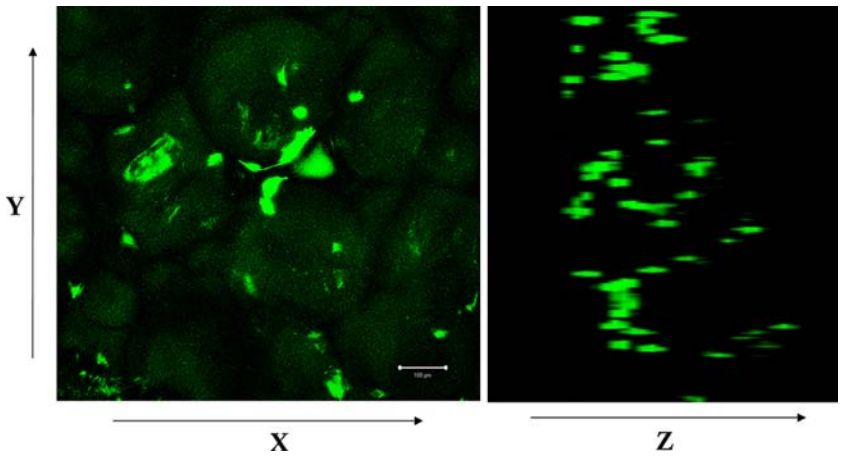

Fig. 6 CLSM *z* sectioning images of cells within 3D porous matrices obtained using gelatine microparticles of 150–300 μm in diameter: **a** *xy* and **b** *yz* projection of 30 overlapped consecutive *z*-slices

remains a challenge in designing tissue engineering scaffolds. Gene delivery can stimulate local protein production able to activate processes that may play important roles in tissue development and physiology [28]. Furthermore 3D structural properties of a scaffold can influence cellular organization and distribution; therefore the functionality of the engineered tissue [8, 29–31].

Combination of the ability to tailor and control scaffolds structure with the capacity of influencing cell fate by gene transfer, has the potential to enhance tissue engineering challenge. To this aim we developed 3D DNA bioactivated PEG hydrogels with well defined pore structure. A preliminary evaluation of the morphological features of hydrogels was assessed by SEM analysis of both samples surfaces and cross sections. All samples are characterised by an extremely interconnected internal porous structure and well-defined porous external surfaces (Fig. 1).

Image analysis investigation has shown a porosity of about 80%. The results of such investigation are affected by the intrinsic error of the used procedure that entails loss of small fundamental details resulting from the use of 2D images to represent solid objects. However, this procedure has proved a valid tool for the description of additional morphological parameters such as pore size and distribution. Pore size coherently varies with particles diameter, given that scaffolds uniformly shrank by ~40%, upon dehydratation (Table 1).

Moreover SEM analyses of samples obtained with different particles dimensions have shown hydrogels with pore dimension gradients (Figs. 2, 3). This is an evidence that the proposed procedure permits to create and tailor porosity and pore size gradients into the matrix. In particular, as expected from the preparation procedure, hydrogels with both two pore dimensions (Fig. 2) and a continuous pore dimension gradient (Fig. 3) have been obtained. Pore size for the former hydrogels has a bimodal distribution with two modes clustering around 80 and 280 μm (Fig. 4). The ability to generate 3D porous matrices with well-controlled anisotropic architectures is highly desirable in designing tissue engineering scaffolds.

Porous scaffolds characterized by pore size gradients offer the great advantage of reproducing the spatial organization of cells and extracellular matrix of highly complex 3D tissues, such as bone and cartilage [32–35].

Quantification of gelatine non specific covalent incorporation in the porous hydrogels has shown that independently from the templating microparticles diameter, 690 ± 170 μg of gelatine are retained in each hydrogels, this quantity represents less then 0.5% of the whole gelatine used to prepare the hydrogels.

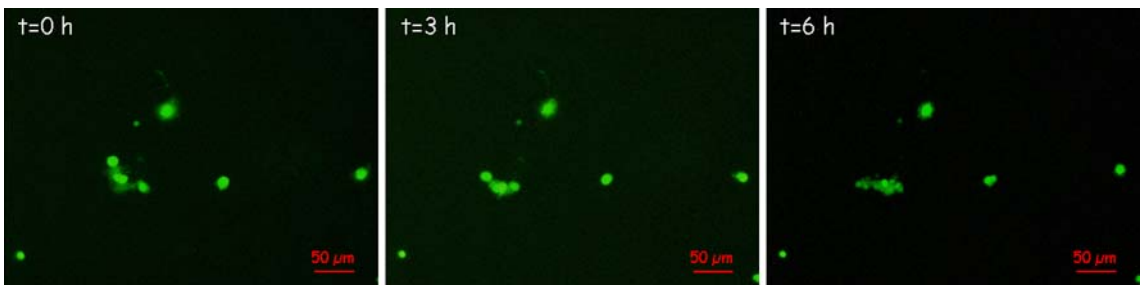

Fig. 7 Time-lapse video microscopy clips spanning 6 h of 3D cell migration into a PEG porous scaffold obtained using gelatine microparticles of 150–300 μm in diameter

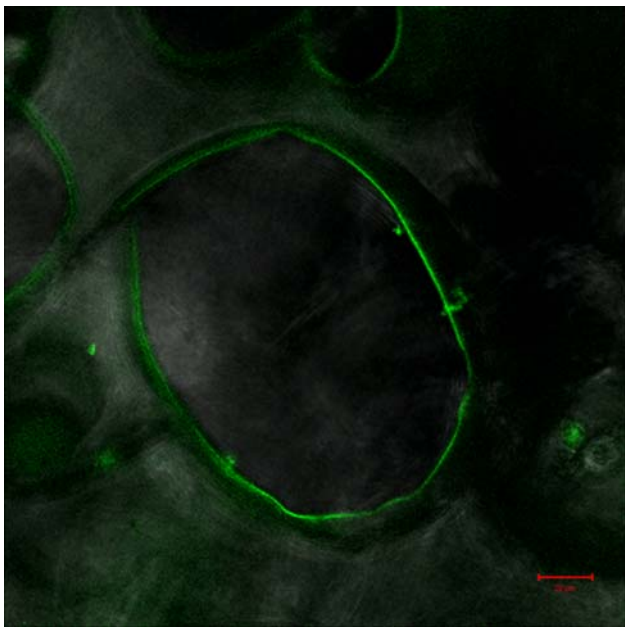

Fig. 8 CLSM picture of PEIpDNA complexes adsorbed on pore surface of a scaffold obtained using gelatine microparticles of 150–300 μm in diameter

The results of CLSM analyses have shown a strong effect of pore dimension on both cell morphology and infiltration [36]. In particular the number of cells able to deeply penetrate the matrices after 72 h of culture increase with pore dimension (Fig. 5). Moreover at the same time point of culture cells appear better spread as the pore dimension increase, indicating a better interaction with the matrix. This last result has been corroborated from *z*-stack analysis performed on scaffolds obtained using templating microparticles of 150–300 μm (Fig. 6), *yz* projections have shown that cells are located at variable focus planes along the *z*-axis and preferentially distributed within matrix pores (Fig. 6b). Additionally, NIH3T3 cells moved inside the 3D scaffold following its micro-architecture (Fig. 7), likely because of combination of serum protein adsorption to PEG and 3D porous structure properties. The Alamar Blue assay has provided that after 22 days of static culture, cells are still viable in all samples. However the rate and trend of cell proliferation are different in the scaffolds with different pore dimension. Alamar Blue assay and CLSM analysis results have indicated that in the sample with the smallest pore dimension, cells colonize prevalently the surface that after 16 days of culture is completely cellularized. While in the sample with the two largest pore dimension (templating microparticles of 150–300 and 300–500 μm) cells completely colonize the scaffolds and after 16 days are confluent. On the contrary in the sample obtained using templating microparticles of 75–150 μm in diameter cells slowly and continuously penetrate the scaffold up to 22 days.

In order to follow the fate of PEIpDNA complexes adsorbed into the PEG scaffolds, fluoresceinated PEI was used. Complexes retention efficiency tests have revealed that the percentage of PEIpDNA complexes adsorbed is 79.1 ± 1, 78.9 ± 3, 75.5 ± 1 and 73.8 ± 2 in hydrogels obtained by using gelatine microparticles of diameters in the 53–75, 75–150, 150–300 and 300–500 μm ranges, respectively. As expected, pore size and number of complexes entrapped in the scaffold, are inversely correlated. CLSM analyses have shown the presence of PEIpDNA complexes on the pore surface of the scaffolds (Fig. 8). Moreover after 48 h of culture, NIH3T3 cells into the matrix were found to express the transgene (GFP).

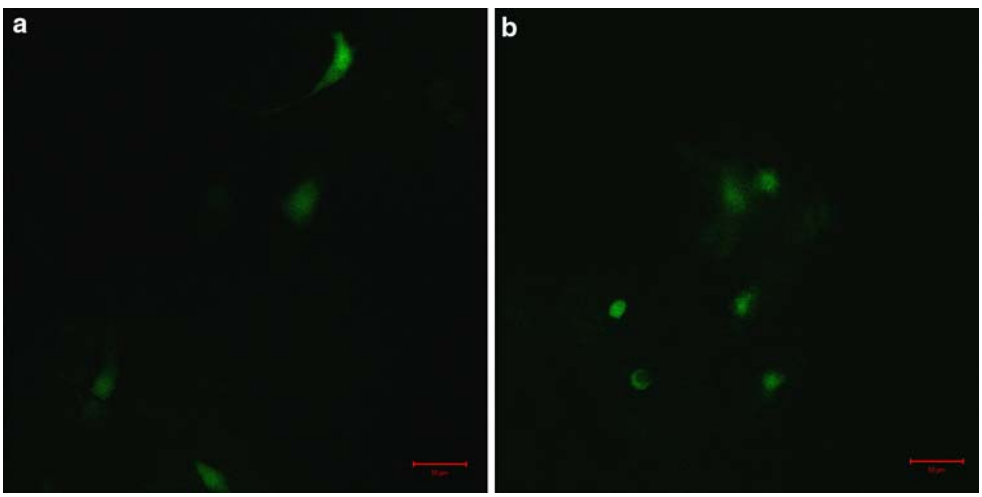

Fig. 9 **a** and **b** CLSM pictures of GFP expressing cells within DNA bioactive 3D PEG porous scaffold obtained using gelatine microparticles of 150–300 μm in diameter after 72 and 96 h, respectively

Additionally, during the course of the experiment (up to 96 h of culture) the number of GFP transfected NIH3T3 cells increased (Fig. 9).

4 Conclusions

The major result of the research carried out is the production of a novel method for preparing gene activated scaffolds with ordered and highly interconnected macroporosity. Using this method, the attractive features of hydrogels (i.e. biochemical versatility, tissue-mimetic mechanical properties, and hydrophilicity), may be combined with the benefits resulting from their induced both ability of influencing cell fate (affecting their processes by DNA incorporation), and interconnected macroporous structure (including improved nutrient transport, and space for cell migration). Furthermore, the elaborated method is a step forward in the production of gene activated matrices with ad hoc microarchitectural features and under very mild physical–chemical conditions.

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