Evaluating the biodegradability of Gelatin/Siloxane/Hydroxyapatite (GS-Hyd) complex in vivo and its ability for adhesion and proliferation of rat bone marrow mesenchymal stem cells

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Abstract Recent studies have shown that the use of biomaterials and new biodegradable scaffolds for repair or regeneration of damaged tissues is of vital importance. Scaffolds used in tissue engineering should be biodegradable materials with three-dimensional structures which guide the growth and differentiation of the cells. They also tune physical, chemical and biological properties for efficient supplying of the cells to the selected tissues and have proper porosity along with minimal toxic effects. In this manner, the study of these characteristics is a giant stride towards scaffold design. In this study, Gelatin/Siloxane/Hydroxyapatite (GS-Hyd) scaffold

was synthesized and its morphology, in vivo biodegradability, cytotoxic effects and ability for cell adhesion were investigated using mesenchymal stem cells (MSCs). The cells were treated with different volumes of the scaffold suspension for evaluation of its cytotoxic effects. The MSCs were also seeded on scaffolds and cultured for 2 weeks to evaluate the ability of the scaffold in promoting of cell adhesion and growth. To check the biodegradability of the scaffold in vivo, scaffolds were placed in the rat body for 21 days in three different positions of thigh muscle, testicle, and liver and they were analyzed by scanning electron microscopy (SEM) and weight changes. According to the results of the viability of this study, no cytotoxic effects of GS-Hyd scaffold was found on the cells and MSCs could adhere on the scaffold with expanding their elongations and forming colonies. The rate of degradation as assessed by weight loss was significant within each group along with significant differences between different tissues at the same time point. SEM micrographs also indicated the obvious morphological changes on the surface of the particles and diameter of the pores through different stages of implantation. The greatest amount of degradation happened to the scaffold particles implanted into the muscle, followed by testicle and liver, respectively.

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Introduction

Incurable diseases or tissue impairs are approached by different ways of tissue or organ transplantation, surgical reconstruction, synthetic prostheses, and medical devices. Each treatment protocol, however suffers from some problems or limitations. For example, organ transplantation is a successful therapeutic approach, but it is challenged by shortage of donors, the necessity for consumption of immunosuppressive drugs and its high potential for severe complications (Fuchs et al. 2001). Although the other therapies are not limited by supply, they also have limitations. For example, synthetic prostheses and medical devices are not able to replace all the functions of a damaged or lost tissue. Tissue engineering has emerged as an expanding approach to address these problems and is a major component of regenerative medicine (Langer and Vacanti 1993; Nerem and Sambanis 1995; Kim and Evans 2005). By combining engineering principles with the knowledge of material science and molecular biology, investigators seek to create novel constructs that will fully integrate into the host system and restore function of the lost tissues (Katz et al. 1999). The general principles of cell-based tissue engineering involve combining the living cells with a natural/synthetic support or scaffold (Stock and Vacanti 2001). With this approach, porous three-dimensional scaffolds play an important role in manipulating the cell functions. Isolated and expanded cells adhere to the scaffold in all three dimensions, proliferate, and secrete their own extra cellular matrices (ECMs), replacing the biodegrading scaffold (Robert 2001; Kim and Evans 2005). Therefore, in addition to permitting cell adhesion, promoting cell growth, and allowing retention of differentiated cell functions, the scaffold should be biocompatible, biodegradable, highly porous with a large surface to volume ratio, able to facilitate the necessary crosstalk that occurs with the surrounding cells, mechanically strong, and capable of being formed into desired shapes with considerable stability (Rahaman and Mao 2005; Choi et al. 2005; Ifkovits et al. 2010; Soliman et al. 2011).

A variety of hydrolytically degradable polymers have been developed for scaffold applications in tissue engineering. However, the majority of these polymers are composed of linear aliphatic polyesters with a high molecular weight, and their copolymers. These

materials often possess mechanical properties best suited for hard tissue engineering (Heller et al. 1990; Freed et al. 1994; Lowry et al. 1997; Ishaug-Riley 1997; Niklason et al. 1999; Agrawal and Ray 2001). However, for engineering of the soft tissues, flexible scaffolds are desirable, since they are amenable to mechanical changes that happen during tissue development (Takahara et al. 1991). The biomaterials used to construct these porous scaffolds include synthetic biodegradable polymers such as poly glycolic acid (PGA), poly lactic acid (PLA), and their copolymer poly DL-lactic-co-glycolic acid (PLGA), and also naturally derived polymers such as collagen and inorganic hydroxyapatite (Freed et al. 1994; Peter et al. 1998; Kim and Mooney 1998). In this study, the GS-Hyd complex was synthesized by combination of Gelatin, Siloxane and hydroxyapatite as will be explained below. Hydroxyapatite, mainly in a carbonated form, is the major inorganic constituent of natural bone (Roseberry et al. 1931; Dorozhkin and Eppe 2002). Synthetically made hydroxyapatite ($\text{Ca}_5(\text{PO}_4)_3(\text{OH})$) has long been used in medicine and dentistry due to its ability to chemically attach to bone. It has been used as a hard tissue replacement, usually in the form of fillers (pastes, powders), or as a bioactive agent in polymer composites (Dorozhkin and Eppe 2002). Pure, stoichiometrical hydroxyapatite is the least degradable form of calcium phosphate (Klein et al. 1990). Often, other more soluble mineral phases of apatite are preferred as bone-substitute materials in order to combine calcium-ion release properties with chemical stability of the support (Daculsi 1998; Legeros et al. 2003; Pasteris et al. 2004). Amongst those are included non-stoichiometrical hydroxyapatite (calcium deficient hydroxyapatite (CDHA)), β -tricalcium phosphate (β -TCP), octacalcium phosphate (OCP), or biphasic hydroxyapatite (BCP, various hydroxyapatite/ β -TCP ratios) (Koerten and van der Meulen 1999; Daculsi 1998; Legeros et al. 2003; Pasteris et al. 2004). A number of synthetic routes towards the preparation of biologically active calcium phosphates, including different templating approaches, have been reported (Sarda et al. 1999; Horvath et al. 2000; Yan et al. 2001; Koumoulidis et al. 2003; Yao et al. 2003). The presence of silica in many biocompatible and bioactive materials has generally shown to improve their bioactivity (Phan et al. 2003; Porter et al. 2004). Studies show that the created or already present silanol groups provide good

nucleation sites for hydroxyapatite (Kokubo et al. 2003). Silica is also one of the vital components in bioglass, but is also used in polymer composites and thin films (Hench et al. 1971; Hench 1991; Ohtsuki et al. 1992; Villacampa and García-Ruiz 2000; Krikorian and Pochan 2003). In addition, natural polymers like collagen, gelatin and chitosan are osteoconductive and biodegradable components that have been used in fabrication of the scaffold materials for tissue engineering (Tenhuisen and Brown 1994; Kang et al. 1999). Thus, hybridization among natural polymers and these inorganic species may yield biodegradable and bioactive scaffolds for tissue engineering (Ren et al. 2001). The scaffolds are normally analyzed by scanning electron microscopy (SEM) for size of the pores, their internal connections and physical appearances. In the scaffold biodegradability process, both in vitro and in vivo, the pore diameters and volume of the scaffolds can be determined by SEM (Silva et al. 2011).

Development of a viable construct also involves a suitable supply of cells that are ideally nonimmunogenic, highly proliferative, easy to harvest and have the ability to differentiate into a variety of cell types with specialized functions (Naughton 2002; Koh and Atala 2004; Van Eijk et al. 2004). One possible candidate for such purpose is the use of BM-MSCs, which have the potential to differentiate into various types of mesenchymal tissues, including osteoblasts, chondroblasts, myoblasts, and fibroblasts (Pittenger et al. 1999). They are present in large quantities in animal and human bodies and can also be easily obtained and propagated in vitro to a large volume (Colter et al. 2000; Jiang and Jahagirdar 2002). Moreover, BM-MSCs are hypoinmunogenic cells that do not induce immune system response (Edalatmanesh et al. 2008) and have been successfully used to promote repair of a number of connective tissues, such as bone (Bruder et al. 1998), cartilage (Fan et al. 2006), tendon (Ouyang et al. 2003) and ligament (Ge et al. 2005). We have also shown in our previous studies, the capacity of the MSCs in regeneration of insulin producing cells and neural cells in animal models of diabetes and Huntington's disease, respectively (Edalatmanesh et al. 2010; Neshati et al. 2010).

In this study, the GS-Hyd complex was synthesized and studied for the three main requirements of an ideal scaffold, including: (a) whether it affects the cell viability, (b) whether it supports the adhesion and

growth of the cells, and (c) whether it is biodegradable in vivo.

Materials and methods

Scaffold preparation

Starting materials used in this investigation for synthesis of the scaffold, including analytical grade calcium nitrate tetra hydrate $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, phosphoric pent oxide (P_2O_5) and tetra ethoxyortho silicate (TEOS) were purchased from Aldrich, Germany. All materials were used as received, without further purification.

Solutions of P_2O_5 (0.5 mol/L (absolute ethanol)) and solution of $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (1.67 mol/L (absolute ethanol)) were prepared. They were mixed together in a Ca/P molar ratio of 1.67 as an initial mixed precursor solution. The mixture was then continuously stirred about 10 min at ambient temperature, followed by heating in a water bath at 60 °C for 1 h. 12.5 wt% solution was prepared by dissolving the gelatin in HCl aqueous solution (0.1 M). Addition of appropriate amount of calcium phosphate to the gelatin solution results in the hybrid solution. After this ripening process, 5 mL TEOS was added to the mixture under continuous stirring, corresponding to 60 wt% SiO_2 in the final SiO_2 /calcium phosphate material. The obtained gel was aged at room temperature for 20 h before calcinations at 550 °C for 8 h (heating rate 1 K min^{-1}), to remove all organics from the material.

Animals

In all experiments, male Wistar rats with an average weight of 250 g were used. Rats were kept at standard conditions subjected to cycles of light and darkness at 12-hour intervals.

BM-MSCs extraction and culture

Bone marrow was obtained from the femurs and tibias of rats. BM was flushed into Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Scotland) supplemented with 15% fetal bovine serum (FBS, Gibco, Scotland) and 100 unit/ml penicillin and 100 µg/ml streptomycin (Biosera, UK) and incubated in a humidified incubator at 37 °C with 5% CO_2 . When cultures

reached about 80% confluency, they were washed with phosphate buffered saline (PBS) followed by a 5 min incubation with 0.05% trypsin solution at 37 °C. Cells were splitted weekly at a ratio of 1:3.

Cytotoxicity assay of GS-Hyd

5×10^3 of MSCs at passage 3 were seeded in each well of 96-well microplates and incubated for 48 h at 37 °C. Then 80 mg of powdered the scaffold was dissolved in 2 ml DMEM, and cells were exposed to various volumes of GS-Hyd suspension (2, 4, 8, 16, 32, 64, 128 and 200 μ l), with three repeats for each volume. Cells without GS-Hyd were used as controls. The effect of GS-Hyd on cell viability was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, Merck, Germany) staining. MTT assay was performed after 48 h of treatments. At desired time points, cells of each well were incubated in 20 μ L MTT solution (5 mg/mL MTT in PBS) for 4 h at 37 °C. The intense purple coloured formazan derivative formed via cell metabolism was then dissolved in 200 μ L/well dimethylsulfoxide (DMSO, Merck, Germany) and the absorbance was measured at 570 nm by ELISA plate reader (AWARENESS, USA). The cell viability for each volume of GS-Hyd against the control was calculated using the following formula: absorbance of each treated well/mean absorbance of control cells $\times$ 100. The average of cell viability for each volume was calculated and the diagram was drawn using Excel program.

Seeding of MSCs on GS-Hyd scaffold

MSCs at passage 3 were trypsinized and suspended in DMEM followed by counting with hemocytometer. They were then centrifuged at 500 rpm for 5 min and the pellet was resuspended in DMEM, supplemented with 15% FBS and 100 units/mL penicillin and streptomycin. 100 μ L medium was used for each 1×10^6 cells. After cutting the scaffolds into small pieces (5 mm $\times$ 3 mm) and sterilization by autoclave, they were placed on agarose-coated (1% agarose in sterile H₂O) 6-well culture plates (3 scaffolds/well), and divided into two test and control groups. The scaffolds without cells served as control group, while each scaffold of those wells which served as test group was seeded with 1×10^6 cells. The scaffolds were incubated for 4 h in a humidified

atmosphere at 37 °C and 5% CO₂ to allow cell attachment. Then 3 mL of the medium was added to each well and the scaffolds were incubated for 2 weeks. The medium was replaced every 2–3 days. On days 1, 7 and 14 the scaffolds were collected to be evaluated for cell attachment by SEM.

Preparation of the cell-seeded scaffolds for SEM

Scaffolds were fixed in 3% buffered glutaraldehyde for 24 h, dehydrated with a graded ethanol series, and dried. The dried samples were mounted on aluminum stubs and sputter-coated (SC7620 sputter coater) with gold for 2 min. SEM was used to take micrographs of the samples. The GS-Hyd scaffold was characterized mainly by fourier transform infrared spectroscopy (FTIR), differential thermal analysis (DTA), and TEM. Simultaneous DTA thermal analyses were carried out in the range of 20–1,000 °C in a Netzsch STA 409 °C instrument under air and at the rate of 10 °C/ min.

Preparation of scaffold for in vivo implantation

Another series of scaffolds were cut in small segments (5 mm $\times$ 3 mm). First, the scaffolds were submerged in 70% ethanol for 30 min, and washed with PBS. The scaffolds were then kept in PBS until the start of the surgery. Scaffolds were polished on the edges to ensure that they were not sharp. The weight of the scaffolds was recorded before the ethanol wash and placement in the PBS.

Preparation of animals for implantation surgery

All tests and experimental protocols were approved by the Ethics Committee of Ferdowsi University of Mashhad. Animals were anesthetized by a mixture of ketamine hydrochloride (100 mg/kg) and xylazine (5 mg/kg). The regions for the surgery (thigh muscle, testicles, and liver) were sterilized after shaving.

For implantation of the scaffold in thigh muscle, a deep incision of 1 cm was made along the length of the muscle with a scalpel. Then tampons were used to stop bleeding. If bleeding was persistent, the site of incision was cauterized. The scaffold was then gently lowered in the muscle fibers. After this step, the muscle was stitched layer by layer towards the surface. To implant the scaffold in testicle, an incision was made after washing and sterilizing of the scrotum. One of the

testicles was exposed, and without applying too much pressure to the testicle, a hole with the exact size of the scaffold was made. The scaffold was then placed in the testicle which was then stitched. For implantation of the scaffold in liver, a deep incision of about 1 cm was made below the right side of the rib cage, until the peritoneum of the liver appeared. Then, a small hole was made in the liver and the scaffold was placed inside. The peritoneum and the skin were stitched separately.

At the end of each surgery, the stitched tissues were sterilized and Lidocaine gel was applied to numb them and avoid the possibility of any infection, every rat was injected intraperitoneally with 5,000 unit/kg penicillin. After these surgery procedures, the animals were moved to a recovery cage to regain their consciousness, in a warm blanket. To ensure that the rat is alive, breathing was checked, the pulse of the rat was taken and the body temperature was monitored. When the vital signs and movements were normal, rats were returned to their original cage.

Degradation assay of GS-Hyd

To analyze the degradability of the scaffold by weight changes, 3 rats were anaesthetized at time points of 3, 7, 16, and 21 days after implantation, and the scaffolds were removed from their bodies. On these days, the anatomical condition and histology of the surgery site were carefully studied. When each scaffold was removed from the body, it was immediately washed with normal saline to remove blood and extra tissues. Each scaffold was placed into a separate test tube with specific identification number. The test tubes were then placed in oven at 50 °C for 30 min. After the scaffolds were completely dried, they were weighed with a sensitive electronic scale. The newly acquired weight for the scaffold was compared to its weight prior to surgery and the percentage of weight loss was recorded. To check the rate of degradation using SEM, scaffold samples from each part of the body (thigh muscle, testicle and liver) were prepared using the same procedure mentioned previously. The amount of connection between the pores of the scaffolds, the diameter of the pores and the dispersion of them were assessed as the marks of degradation rate.

Statistical analysis

Data were analyzed using one way ANOVA. All group comparisons were analyzed using Tukey's post

hoc test to determine which groups were significantly different from each other. Statistically significant data were considered at the $P \leq 0.05$ level.

Results

Structural characterization of the scaffold

GS-Hyd scaffold showed sharp absorption bands in FTIR (Fourier Transformed Infrared Spectroscopy) that were attributed to hydroxyapatite: 3th vibration of PO_4^{3-} ($\nu_3\text{PO}_4^{3-}$) at 1,045–1,092 cm^{-1} and $\nu_2\text{PO}_4^{3-}$ at 474 cm^{-1} , $\nu_1\text{PO}_4^{3-}$ symmetric stretch at 963 cm^{-1} , $\nu_4\text{PO}_4^{3-}$ bending at 602 and 572 cm^{-1} . The broad absorption bands around 3,100–3,600 cm^{-1} belong to OH stretching absorption bands. Extensive absorption is seen around 630 cm^{-1} (structural OH stretching), attributed to crystalline structure of hydroxyapatite. The silica layer shows weak absorption bands around 800 cm^{-1} corresponding to the Si–O–Si bonds. Some of the silica absorption bands are hard to identify due to stronger overlapping absorption bands from hydroxyapatite. That is especially true in the area around 1,185 and 1,085–1,000 cm^{-1} where the Si–O–Si vibration bands usually are the strongest, but also around 960 cm^{-1} where the Si–OH silanol groups absorb (data not shown).

Figure 1 shows, the thermogram corresponding to simultaneous DTA thermal analyses dried at 70 °C/24 h. In the DTA curve of the precursor GS-Hyd scaffold (Fig. 1), after dried in 70 °C, exothermic peaks assignable to organic combustion are visible. In order to discard the presence of water occluded in the exhaustively dried sample after 150 °C, a simultaneous DTA analysis was carried out due to its considerably higher sensibility. DTA showed the combustion peak of residual gelatin at 320 °C, and a very low peak at 590 °C, it can be concluded that the microporosity observed after calcination is originated by the elimination of gelatin macromolecules forming microdomains in the silica matrix.

The simple one-pot synthesis method allows a fairly homogeneous distribution of the mesoporous silica throughout the apatite crystal surface, which is demonstrated in the TEM micrograph (Fig. 2). The crystalline size of GS-Hyd scaffold is about 50 nm. This could be the reason for well distribution of hydroxyapatite in the surface of silica matrix. On the other hand, the material lacks a well-pronounced mesoporous long-range order in the silica layer.

Fig. 1 DTA thermogram of GS-Hyd

Fig. 2 TEM image of GS-Hyd showing the great growth of hydroxyapatite and placing the silica among the pores of it

Fig. 3 The control SEM micrograph showing the morphology of the scaffold without cells

Homing of MSCs in the scaffolds

As mentioned previously, 1×10^6 cells were seeded on each scaffold of the test group and scaffolds without cells served as negative controls (Fig. 3). On days 1, 7

and 14 after cell seeding, scaffolds were collected to be evaluated for cell attachment and proliferation by SEM. Cells were in round shape at the day of seeding (not shown) but could adhere on the scaffold, expand their elongations and form fibroblast-like

Fig. 4 SEM micrograph showing scattered distribution of MSCs on the surface of the scaffold on the first day of cell seeding

Fig. 5 SEM micrograph showing MSCs proliferation and colony formation on the scaffold, 7 days after the seeding

cells within 24 h (Fig. 4). Cells were proliferating during the cultivation on scaffolds, so that they could form colonies after 7 days of their seeding (Fig. 5).

Fig. 6 Effects of different volumes of GS-Hyd suspension on viability of MSCs as measured by MTT assay

Cytotoxicity assay of GS-Hyd

The MTT assay is a colorimetric approach for measuring the activity of enzymes that reduce MTT to formazan dyes, giving a purple color. A main application allows assessing the viability and the proliferation of cells. It can also be used to determine cytotoxicity of potential medicinal agents and toxic materials, since those agents would stimulate or inhibit cell viability and growth.

MTT results showed that GS-Hyd has no detectable cytotoxic effects on the cells and cell viabilities under different volumes of the GS-Hyd were similar to that of the controls (100%). However, the cell viability decreased only in 200 μl of GS-Hyd as shown in Fig. 6.

Analyzing degradability of the GS-Hyd scaffold in vivo

The scaffold weight changes at each position were recorded for different consecutive times of 3, 7, 16 and 21 days after surgery. The weight change differences between the 3rd day were significant with the 16th and the 21st day, but the differences between other days were not significant. The differences between thigh and liver ($P < 0.0001$) and between liver and testicles were significant ($P < 0.001$), but there was not significant difference between thigh muscle and testicle (Fig. 7).

Structural changes of the scaffolds following in vivo implantation

SEM micrographs indicated the obvious morphological changes on surface of the particles through

Fig. 7 Comparison of the rate of scaffold degradation in various anatomical positions on different days after implantation. There are significant differences in each group during the time period, but this difference was not significant between groups at the same time point. Vertical bars indicate means $\pm$ SEM in rats of each group at each measurement ($n = 3$, $P \leq 0.05$)

different stages of implantation (Fig. 8). Moreover, a considerable change happened in diameter of the pores during the assessment time. In comparison, it seemed that the highest to lowest rate of degradation belonged to particles in thigh, testicles and the liver on day 21 post implantation (Fig. 8, b–d) respectively.

Discussion

One of the main problems in cell therapy is homing or maintaining the cells in desired areas, which may be overcome by growing the cells on scaffolds and their transplantation at the target sites. The major concerns in application of the external scaffolds in tissue engineering are severe immune response, persistence to degradation, and unexpected side effects. As mentioned before, (1) the specific material composition of the scaffold and (2) the cell source are two important elements, which can substantially affect the outcome of tissue engineering (Katz et al. 1999). Application of biodegradable materials is a vital factor in tissue engineering (Xie et al. 2007). Scaffolds should also be mechanically strong, capable of being formed into desired shapes, non cytotoxic and highly porous for permitting cell adhesion and growth

(Rahaman and Mao 2005; Choi et al. 2005). Development of such an efficient scaffold also involves a suitable supply of cells such as MSCs which their unique properties make them a superior candidate over other cells for clinical purposes. For example, Liu and his colleagues cultured anterior cruciate ligament fibroblasts (ACLFs) and BM-MSCs on the combined silk scaffolds to investigate the biological cellular responses that can be used to determine the most appropriate cell sources for ligament tissue engineering application. Their preliminary results demonstrated that BM-MSCs have much clearer and distinct advantages over ACLFs, with respect to cell proliferation and in vivo survival (Liu et al. 2008). Meanwhile, there are less concerns about tumor formation and ethical problems for MSCs. Although some studies have shown a promotion for tumor growth and metastasis after implantation of MSCs, tumor induction is mostly described for embryonic stem cells (ESCs) (Arnhold et al. 2004; Tang et al. 2003).

Cell adhesion to substrate is known to affect cell behaviour and functions in both natural and engineered tissues and plays a key role in morphogenesis and organogenesis (Thiery 2003; Lakard et al. 2004). In this study, we used MSCs as candidate stem cells and GS-Hyd scaffold as a cell carrier for adhesion and proliferation of the cells in vitro. GS-Hyd is a three-dimensional scaffold which did not show any cytotoxic effects and preserved the cell viability in vitro. GS-Hyd also promoted cell adhesion and proliferation. Considering the fact that cells were in round shape at the day of seeding (not shown) but could expand their elongations and form fibroblast-like cells within 24 h (Fig. 4), it was shown that MSCs had adhered to the GS-Hyd scaffold very well. As the initial number of the cells on all scaffolds was the same and more cells were visible on day 7 rather than day 2, it could also be concluded that the number of cells increased during the cultivation on scaffolds and MSCs could form colonies after 7 days of cell seeding (Figs. 3, 4 and 5). The interconnected pores of the scaffold may result in cellular adherence. Additionally, the pore size (1–2 μm) of the scaffolds seems to be large enough to allow for easier medium flow which would provide ideal conditions for cell proliferation on the scaffolds.

Composition of the scaffold and the speed of the scaffold degradation in its biological environment, are vital factors determining the rate of scaffold

Fig. 8 SEM micrographs of the scaffold before (a) and after (b–d) implantation in the animal body. b–d letters represent implantation sites of the liver (b), testicle (c) and thigh muscle (d)

degradation. The rate of the scaffold degradation is also a concern in its application in vivo. However, this is not the case for GS-Hyd scaffold, because its rate of degradation can be manipulated during the synthesis. In previous studies on similar scaffolds, they were kept in the body of rats for duration of 30–90 days after surgery (Kim et al. 2006; Piao et al. 2007). In our study, we implanted and kept the scaffolds for 21 days. During this period, the speed of degradation in the beginning of shortly after scaffold implantation was much higher than at the last days. This could be explained by the fact that gelatin, the main component of the scaffold, is easily degraded and could contribute to the major weight lost in the beginning. However, more proper experiments should be designed to address this matter precisely. At the end of the period about 30% of the scaffolds remained intact. Precise measurements showed considerable weight changes

during the period of implantation and these volume changes were significant for all sampling days. Therefore, it seems that the scaffold is probably degraded due to disappearance of the interconnections of pores inside the scaffold. The enlarging of the pores, in diameter, was confirmed by SEM. This fact shows a clear connection between compound degradation and inter-junction loss. Structural degradation rates at the three examined anatomical conditions proved that the degradative capacities are tissue specific and according to our SEM micrographs the thigh muscle has the highest capacity for the scaffold degradation. During the whole period of experiments, there were no other signs of any toxic effect on the rats, other than a slight inflammation.

In conclusion, the capacity of GS-Hyd for degradation in vivo with acceptable rate was confirmed in this study. It was also shown that GS-Hyd did not have

any cytotoxic effects and also could promote cell adhesion and proliferation in vitro, but to reach to a definite conclusion about the toxic effects of the scaffold in vivo, further experiments are needed. Therefore, we suggest that GS-Hyd scaffold can be used as a potential structure for tissue engineering, but still more assays and in vivo studies are required to investigate other characteristics of this scaffold which are important in tissue engineering in order to evaluate its advantages for clinical purposes.

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