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In vitro characterization of chitosan scaffolds: influence of composition and deacetylation degree

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Abstract In this study, the influence of degree of deacetylation (DD) and composition on some structural and biological properties of chitosan scaffolds were examined in vitro. 3D chitosan scaffolds of 2% (w/v) and 3% (w/v) composition in different DDs i.e. 75–85% and >85% were prepared by freeze-drying method at –80 °C. We noticed that >85% deacetylated chitosan scaffolds of 2% (w/v) composition has a highly interconnected morphological structure having ~100 μm pore size with 0.0917 N/mm^2 compression modulus. L929 fibroblastic cells were cultured on chitosan scaffolds in order to evaluate their biocompatibilities. Cell culture studies demonstrated that fibroblastic cell attachment and proliferation is affected by DD. The higher deacetylated chitosan scaffolds strongly supported the attachment and proliferation when compared with the lower deacetylated scaffolds. MTT assay indicated that >85% deacetylated chitosan scaffolds of 2% (w/v) composition, having the highest specific growth rate 0.017 h^{-1} of all, was found to be the most suitable for cell culture studies and a potential candidate for tissue engineering with enhanced biostability and good biocompatibility.

Introduction

Chitosan is a biocompatible and biosorbable biopolymer which is currently receiving a great deal of interest for medical and pharmaceutical applications due to its interesting intrinsic properties [1, 2]. Regarding to its high biocompatibility, it is employed in various implantable and injectable systems such as orthopedic/periodontal composites [3], drug delivery systems [4], wound healing management [5] and scaffolds for soft and hard tissue regeneration [6–8]. It is a bioreactive polymer which behaves as a hemostatic agent presenting antithrombogenic properties [9] and stimulates the immune system of the host against viral and bacterial infections [10]. Moreover, chitosan is easily hydrolyzed and metabolized by various chitosanases and lysozyme and considered as biodegradable. The biodegradation leads to the release of aminosugars which can be incorporated into glycosaminoglycans and glycoproteins metabolic pathways and excreted [11]. Chitosan has the ability to elicit specific cellular functions by inducing the release of cytokines that favor the histoarchitectural organization of connective tissues. It was found to improve osteogenesis and angiogenic activity [8] and showed promising properties for hepatocyte culture/transplantation [12], articular cartilage tissue [13] and skin tissue regeneration [6].

Chitosan is a copolymer of β-[1 → 4]-linked N-acetyl-D-glucosamine and D-glucosamine units. It is obtained by alkaline deacetylation from chitin, which is the main component of the exoskeleton of crustaceans, such as shrimps. Despite numerous attempts, the N-acetyl groups can not be completely removed from chitin [14]. As a result chitosan always contains a number of N-acetyl-D-glucosamine units and the proportion of these units with respect to the total number of units (N-acetyl-D-glucosamine units plus D-glucosamine units) is represented as the ''degree of acetylation'' (DA). It is possible to change the deacetylation degree (DD) of chitin by changing the conditions of deacetylation reaction, in order to prepare various chitosans which have different DA. In the case of chitosan, DA is considered to be below 50% and it is a structural parameter

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influencing the solubility, crystallinity, charge density and enzymatic degradation rate of chitosan [14]. Additionally, cell adhesion and proliferation of certain cell types like keratinocytes, fibroblasts and rat bone marrow stromal cells were reported to be influenced by the DA [11, 15].

Recent studies in regenerative tissue engineering are focused on the use of biodegradable scaffolds to support and organize the damaged tissue since three-dimensional matrices provide a more favorable ambient for cellular behavior. Porous collagen sponges [16], poly-lactic-glycolic acid copolymer and poly-ε-caprolactone [17, 18] were investigated as tissue supporting scaffolds. With its low immunogenic activity, controlled biodegradability and porous structure, chitosan scaffolds are promising materials for the design of tissue engineered systems [6, 7, 19]. In various studies, porous chitosan scaffolds have been prepared by controlled freezing and lyophilization [19], freeze-gelation [7] and crosslinking [20] and possible use in tissue engineering applications have been investigated.

In the literature a few reports are available to indicate the influence of acetylation degree on the cellular responses of chitosan films [11, 15]. However, there is no study describing the effects of DA (or DD) on the biological properties of 3D chitosan scaffolds. In this study, we used two types of commercially available chitosan which are prepared from chitin in different DDs i.e.75–85% and >85. The porous scaffolds were prepared from these chitosan materials by using freeze-drying method and their in-vitro characterization was performed by microscopic observations (optical, Scanning Electron Microscopy (SEM) and Confocal Laser Scanning Microscopy (CLSM)), mechanical tests, biodegradation tests and cell culture studies.

Materials and methods

Materials

Chitosans used in the present work were purchased from Sigma-Aldrich Chemical Co. with deacetylation degrees of >85% and 75–85%. The interval of molecular weights of >85% and 75–85% deacetylated chitin determined by the manufacturer are 190–375 kD and 190–310 kD, respectively. Acetic acid (HPLC grade), ethanol (96% v/v) and chicken egg white lysozyme (~50,000 units/mg) were obtained from Sigma Co. (Germany). All solvents are of analytical grade. Ethidium bromide, glutaraldehyde and hexamethyldisilazane were purchased from Sigma Co. (Germany).

Preparation of chitosan scaffolds

Chitosan solutions with concentrations of 2 and 3% (w/v) were prepared by dissolution in 0.2 M acetic acid and were filtered through 0.45 μm filter (Milipore) in order to eliminate the impurities. Porous chitosan scaffold samples were prepared by freeze-drying method. In brief, chitosan solutions were poured into 24-well tissue-culture polystyrene dishes (TCPS, TPP Switzerland), having a diameter of 1.5 cm, to a depth of approximately 0.5 cm and frozen at –20 °C for 24 h. Then they were transferred into freeze-drier (Christ, Germany) and lyophilized at –80 °C for 4 days to ensure that they were completely dried. The size of lyophilized scaffolds are 14 mm diameter and 4 mm thickness.

Stabilization of chitosan scaffolds

Freshly lyophilized scaffolds were rehydrated by ethanol in order to stabilize the structure. This is why, they were immersed in 96% (v/v) ethanol for 1 h then in 70% (v/v) ethanol for overnight.

Microstructural characterization of chitosan scaffolds

Pore size distribution

Pore size distribution of porous scaffolds was examined by using Mercury Intrusion Porosimetry (MIP, Quantachrome, USA). MIP is based on the premise that a non-wetting liquid, such as mercury, will only intrude pores under pressure. Tests were performed under low pressure conditions in the range of 0–50 psi. The contact angle of mercury on chitosan is 140°, and mercury surface tension is 480 erg/cm^2.

SEM analysis

Surface and cross-section morphologies of lyophilized chitosan scaffolds were examined by using a JEOL (JSM-840A, Japan) SEM after coating with a gold-palladium layer.

Swelling studies

Stabilized chitosan scaffolds were fully rehydrated in PBS (Phosphate Buffer Saline, pH 7.4) at 37 °C in order to investigate the swelling characteristics. Samples were taken out of the buffer solution and the excess of buffer was removed by blotting with filter paper. Swelling ratios were determined gravimetrically by using the following equation (Eq. 1):

$$\text{Swelling ratio} = (W - W_0)/W_0 \quad (1)$$

where W_0 is dry weight and W is wet weight of chitosan scaffold.

Mechanical testing of chitosan scaffolds

Test samples of having a 2 mm thickness were fully rehydrated in PBS (pH 7.4) at 37 °C for 1 h before material testing. Compression tests were performed at constant cross head speed of 5 mm/min with a load of 500 N cell (Lloyd Ins., UK). The compression modulus was calculated from the slope of the linear region in the stress-strain curves for each type of chitosan scaffold.

Biodegradability

In order to investigate the degradation of chitosan scaffolds in vitro, rehydrated scaffolds prepared from 75 to 85% DD with 2% (w/v) and >85% DD with 2% (w/v), having 2 mm thickness, were incubated in 2 ml of complete culture medium (DMEM, Sigma Co.) with 0.1% (w/v) sodium azide (Sigma Co.) containing 500 μg/ml chicken egg white lysozyme. The scaffolds were incubated in complete culture medium without lysozyme for control. Incubations were performed in a 37 °C humidified incubator for 21 days and media was renewed weekly. Dry weights of dehydrated control and test scaffolds were measured at 1st, 4th, 7th, 14th and 21st days of incubation. Scaffold weights were then recorded and compared to their initial dry weight. Degradation was determined as the percentage of weight loss (W_L) according to the Eq. 2:

$$W_L\% = [(W_0 - W)/W_0] \times 100 \quad (2)$$

where, W_0 is initial weight, and W is weight after degradation.

Cell culture studies

Cell line and maintenance

Cell culture studies were carried out with L929 mouse fibroblasts. L929 cell line was obtained from HUKUK Cell Line Collection (No: 92123004, Foot and Mouth Disease Institute, Ankara, Turkey). The cells were subcultured in flasks using Dulbecco's modified Eagle's medium (DMEM, Sigma Co., Germany) supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma Co., Germany). The cells, maintained at 37 °C in a humidified CO_2 (5%) atmosphere (Heraus Instruments, Germany), were dissociated with 0.01% trypsin/10 mM EDTA (Sigma,Germany), centrifuged and resuspended in medium prior to cell seeding.

Cell culture and seeding on chitosan scaffolds

Cell cultures were conducted in parafilm coated 24-well TCPS dishes in stationary conditions. The parafilm coated wells were soaked in 96% ethanol and placed under UV light for 30 min for sterilization. Chitosan scaffolds, having 9 mm diameter and 1 mm thickness, were sterilized with 70% ethanol for 24 h and equilibrated in sterile Dulbecco's PBS (pH 7.4, 24 h). Finally, scaffolds were immersed in conditioning medium for 24 h prior to cell seeding. 1.5 ml of cell suspension containing 3.4×10^4 cells/ml were then added to each well to inoculate the cells on chitosan scaffolds.

Cell attachment

The number of cells diffused to the chitosan scaffolds were determined by hemocytometric countings. At different culture times (1, 2, 4 and 6 h), the non-adhered cells in the medium were counted with a Neubauer hemocytometer by using trypan blue exclusion method. Cell diffusion into scaffolds was determined by subtracting the counted cells from the total cells seeded. The growth index was determined from MTT assay results at the end of first day since only attached cells remain viable on scaffolds.

Cell growth kinetics and fibroblastic cell proliferation

Proliferation of fibroblastic cells on the scaffolds were quantitatively assessed with 3-[4,5-dimethylthiazol-2-yl]-diphenyltetrazolium bromide (MTT) formazan at different culture periods up to 6 days. At different culture times, 0.75 ml culture medium was aspirated and replaced with 0.75 ml fresh medium, then 0.15 ml MTT solution (2.5 mg/ml MTT dissolved in PBS) was added. The samples were incubated at 37 °C for 4 h. Thereafter, the medium was removed and 1.5 ml of 0.04 M HCl in isopropanol solution was added to dissolve formazan crystals and incubated at 37 °C for 1 h with a rotary agitation at 60 rpm. The absorbance was measured by spectrophotometer at 540 nm using a Labomed Double Beam UV-visible spectrophotometer (USA). MTT assay was also applied to the scaffolds without cells and the data was expressed as control. Exponential cell growth was assumed and a specific growth rate (μ) was determined by fitting the following equation (Eq. 3) to the absorbance data:

$$\ln(A/A_0) = \mu(t - t_0) \quad (3)$$

where A_0 is the initial absorbance at t_0 (h), A is the absorbance at t (h) and μ is the specific growth rate (h^{-1}).

Microscopic imaging of cells within scaffolds

The morphology of the cells in the scaffolds was observed by a SEM (JEOL JSM-840A, Japan) at the end of four days of incubation. The scaffolds were gently washed with PBS

and cells were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M PBS (pH 7.4) for 1 h at 4 °C. Then the scaffolds were dehydrated in ethanol series (30%, 50%, 70%, 90%, 96% (v/v)) and rinsed with hexamethyldisilazane. The samples for second and fourth days of incubation period were prepared for visualization with fluorescence (Olympus, USA) and confocal laser scanning microscope (CLSM, Leica, Germany). Briefly, scaffolds were washed with PBS and fibroblasts were fixed with the same procedure as described above. Fixed cells were stained with 0.5 μg/ml ethidium bromide in PBS for 15 min in the dark at room temperature and examined by fluorescence and CLSM.

Results and discussion

Fabrication of porous chitosan scaffolds

Porous chitosan scaffolds with different DDs and compositions were prepared by freeze-drying method. In this method, chitosan solutions were frozen to maintain ice crystals after gelation was occurred and freezing chitosan solutions were lyophilized in order to maintain porous and interconnected structure by removal of ice crystals from the frozen solutions. All scaffolds were subsequently formed from refrigerated solutions without crosslinking to increase biodegradability and to achieve superior biocompatibility for cell culture studies.

Chitosan functionality depends upon the degree of deacetylation (DD) of chitin which in turn depends on the preparation procedure [21]. There is a just opposite relationship between DD and DA. In other words, high DD of chitin leads to low degree of acetylation (DA) for chitosan. This is why, here chitosan scaffolds with 75–85% DD indicates the chitosan which has relatively high DA while chitosan >85% DD is indicating the relatively low DA.

The morphological structure of stabilized chitosan scaffolds is shown in Fig. 1a–h. Surface and cross-section SEM images of chitosan scaffolds with different DDs showed not only a complete porous morphology but also an interconnected structure was observed for all types of scaffolds. However, it was observed that chitosan scaffolds with low deacetylation degrees (75–85%) showed a more regular structure and the pores were fairly uniform and parallel with a polygonal cross section (Fig. 1a–d). In this geometry, lateral pore connectivity appeared to be much lower than for scaffolds with high deacetylation degrees (>85% DD) (Fig. 1e–h). In addition, higher solution concentration produced lower connectivity (Fig. 1c, d, g, h). It is known that the microstructure such as pore size, shape and distribution, has prominent influence on cell intrusion, proliferation and function in tissue engineering. The pore sizes of scaffolds were calculated from cross-section SEM images in Fig. 1. According to these images, the pore sizes of chitosan scaffolds of 2% (w/v) and 3% (w/v) compositions were found to be ~100 μm and 20–80 μm, respectively. Bigger pores being formed at lower concentrations, may be due to the formation of large ice crystals from dilute chitosan solutions. It must be noted that, pore diameters of all scaffolds increased significantly with the radial distance from the edge to the center of the sample, due to the temperature gradient during fabrication. Madihally and Matthew showed that, pore size and connectivity of chitosan scaffold are influenced by the freezing temperature, concentration of chitosan solution and distance from the vessel wall [19].

Stabilization of chitosan scaffolds

Freshly lyophilized scaffolds were stiff and inelastic. As lyophilized structure was composed of soluble chitosan acetate, the chitosan scaffold would exhibited rapid swelling and ultimately dissolved when rehydrated in a neutral aqueous medium. It is reported that scaffolds were stabilized by either dilute NaOH or in an ethanol series [19]. Hydrated scaffolds in NaOH exhibited some shrinkage and distortion, probably caused by base-induced changes in crystallinity and associated structural stresses [17]. Therefore, in this study, stabilization was performed by rehydrating samples in 96% (v/v) ethanol for 1 h and then in 70% (v/v) ethanol for overnight in order to prevent scaffold dissolution. It was observed that samples hydrated through ethanol exhibited no significant changes in shape, they were spongy and flexible and a controlled swelling was performed without dissolution (Fig. 2).

Swelling properties

Swelling studies were performed by rehydrating samples in PBS (pH 7.4) at 37 °C. The results are shown in Table 1, indicating that little variation in water absorption was observed for all types of chitosan scaffolds with equilibrium swelling ratios in the range of 40–27. The swelling ability of the chitosan scaffolds could be attributed to both of their hydrophilicity and the maintenance of their 3D-structure. As it is seen from Table 1, swelling ratios of chitosan scaffolds of 3% (w/v) composition were lower than that of other scaffolds prepared from 2% (w/v) composition since the pore sizes of those were relatively smaller and pore interconnectivities were lower.

Since deacetylation of chitin converts the acetamide group into a primary amine group, which is generally more hydrophilic than its acetamide, increased water content of the scaffolds prepared with high DD is expected. However, we could not find a relationship between DD and swelling

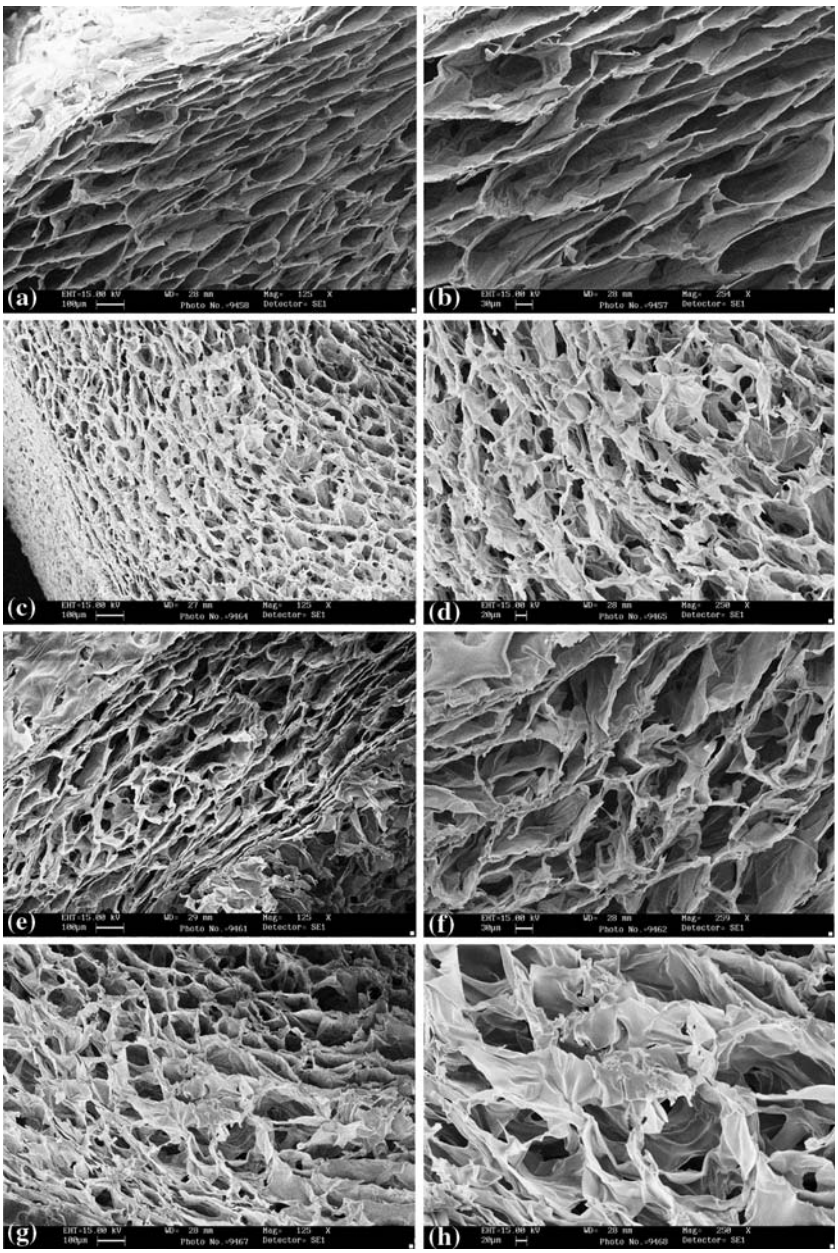

Fig. 1 The cross section SEM images of lyophilized scaffolds at different magnifications. 2% Chitosan (75–85% DD); (**a**) ×125; (**b**) ×250, 3% Chitosan (75–85% DD); (**c**) ×125; (**d**) ×250, 2% Chitosan (>85% DD); (**e**) ×125; (**f**) ×250, 3% Chitosan (>85% DD); (**g**) ×125; (**h**) ×250

ratio. Tomihata and Ikada observed the similar results and they explained that, swelling of deacetylated chitin derivatives is also governed by factors other than the content of the acetylated hydrophobic group in the molecules [22]. The swelling process realized rapidly and the state of equilibrium was reached within 3 min.

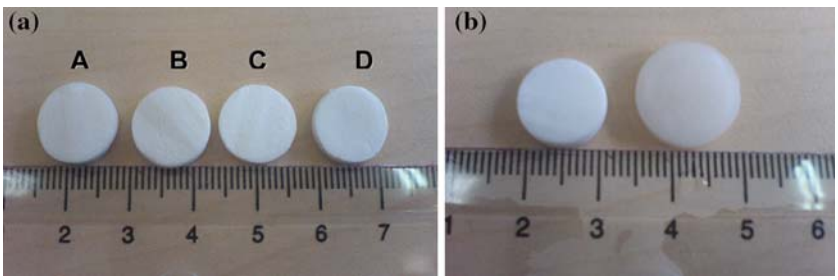

Fig. 2 General view of chitosan scaffolds (**a**) Freshly lyophilized scaffolds; (**b**) Comparison of stabilized scaffold (*right side*) and swollen scaffold (*left*)

Table 1 Swelling characteristics of chitosan scaffolds

Chitosan type		Equilibrium swelling ratio*
A	2% Chitosan (75–85% DD)	40.6 ± 0.7
B	3% Chitosan (75–85% DD)	27.0 ± 1.4
C	2% Chitosan (>85% DD)	36.7 ± 0.2
D	3% Chitosan (>85% DD)	32.3 ± 0.4

* statistically 95% confidential level

Porosity of chitosan scaffolds

Mercury intrusion porosimetry was used to determine the pore size and size distribution of scaffolds. The results are shown in Fig. 3, indicating that most pores are in the range of 30–150 μm, demonstrating that the pore-size information obtained from SEM is reliable, and this porosity was expected to satisfy the cell penetration as well as mass transport requirements for nutrient, metabolites, and soluble signals [21, 23].

Mechanical testing of chitosan scaffolds

The mechanical properties of porous scaffolds are of particular concern for many tissue engineering applications due to the necessity of the structure to withstand stress during culturing in vitro and as in vivo implants. Mechanical properties also influence specific cell functions within the engineered tissues [24, 25]. Thus, in the present study, the compressive properties of scaffolds, which have different DD and compositions, were tested in wet conditions. In general, elastic polymer materials exhibit two characteristic regions of deformation in their stress-strain curves. At lower strains, the stress increases rapidly with increasing strain. Steep initial slopes can be observed in the elastic region and quantitatively give an indicator for higher elastic modulus. At higher strains, the stress only displays a slight increase with increasing strain until failure occurs, reflecting the ductile property of the material [26].

The chitosan scaffolds in hydrated form were soft, spongy and flexible. Figure 4 illustrates typical stress-strain curves for all scaffolds used in this study. As it is seen from this figure, the chitosan scaffolds, which have 3% (w/v) composition, displays a relatively stiff behavior due to the rapid increase in stress region at lower strain values. When Compression Modulus was calculated (Table 2), it was observed that chitosan composition decreased the stiffness and a significant decrease was observed in chitosan scaffolds with lower compositions. This may be due to the difference in porosities between the scaffolds. A higher pore volume and a larger pore size inside the hydrated scaffold provide the scaffold with a larger deformable space. Thus, the scaffolds of 3% (w/v) composition, although having small pore sizes, and the scaffolds with higher DD are mechanically desired scaffolds of all. However, according to compression module values, mechanical strength of chitosan scaffolds, having same compositions, were relatively high due to the increase of DD. The same relation between mechanical strength and DA was already indicated by other researchers [11]. They showed that brittleness increases with DA.

Degradation characteristics of chitosan scaffolds

Biodegradability is a major property for tissue engineering applications due to tissue formation. It was known that chitin and its deacetylated derivatives undergoes lysozyme-dependent degradation [25] which depends on the DD, local pH and source of chitin. The previous studies showed that lysozymal degradation decreases with increase in DD due to the lack of acetyl groups which are necessary for lysozymal binding. In this study degradability of chitosan scaffolds with different DDs were investigated. According to the results, scaffolds incubated in DMEM alone and incubated in lysozyme showed no significant weight changes up to 7 days. After 3 weeks of incubation period enzymatic degradation effect was observed for both types

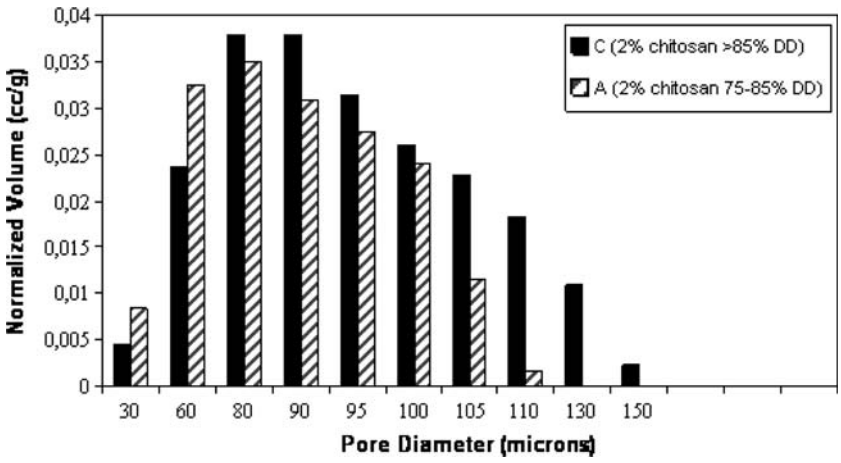

Fig. 3 Pore size distributions of 2%(w/v) chitosan scaffolds in different DDs obtained from MIP

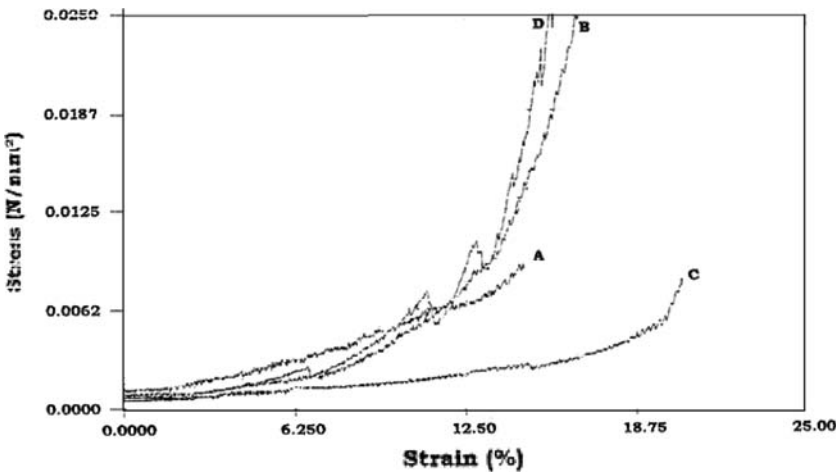

Fig. 4 Compressive stress-strain curves of chitosan scaffolds

Table 2 Stiffness of chitosan scaffolds

Chitosan type		E modulus* (N/mm^2)
A	2% Chitosan (75–85% DD)	0.0572 ± 0.0082
B	3% Chitosan (75–85% DD)	0.0983 ± 0.0080
C	2% Chitosan (>85% DD)	0.0917 ± 0.0001
D	3% Chitosan (>85% DD)	0.1662 ± 0.0464

* statistically 95% confidential level

of chitosan scaffolds. Scaffolds incubated in lysozyme with lower DDs are showing more than 20% weight loss, the same scaffolds showed approximately 10% weight loss in the absence of lysozyme.

Cell culture on chitosan scaffolds

Effect of deacetylation degree on cell attachment

After a certain amount of cell concentration (3.4×10^4 cells/ml) were seeded on different types and compositions of scaffolds, cell attachment studies were performed in order to indicate a complete cell diffusion into scaffolds. The results, determined by hemocytometric counting, showed that L929 fibroblasts were completely diffused to all types and compositions of scaffolds at the end of 6 h. As only attached cells remain viable on scaffolds, growth index on scaffolds was determined from MTT assay at the end of 24th-h of incubation period (Table 3). High growth index values were observed on scaffolds which have high DDs. Increasing DD leads to cationic polymers carrying primary amine groups at neutral pH. Thus, cells attach to scaffolds by nonspecific electrostatic interactions occurring directly between protonated amine groups and surface of cell membrane. Since it was reported that lower degree of acetylation (DA) favoring cell adhesion [11, 15], the results of our study which are based on DD were in good correlation with the studies in literature.

Cell growth kinetics and fibroblastic cell proliferation

The viability of fibroblasts on chitosan scaffolds with different DDs and compositions were evaluated through 5 days of incubation and the results were given as the optical density measured by MTT assay (Fig. 5). Control data are obtained from the scaffolds without cells. After

Table 3 L929 fibroblastic cell growth and attachment on chitosan scaffolds

Chitosan type		Specific growth rate (μ, h^{-1})	Doubling time (t_d, h)	Growth index at the end of 24th-h	Growth index at the end of the exponential phase
A	2% Chitosan (75–85% DD)	0.009	77.0	1.80 ± 0.53	3.62 ± 0.29
B	3% Chitosan (75–85% DD)	0.012	57.7	1.55 ± 0.49	3.71 ± 0.75
C	2% Chitosan (>85% DD)	0.017	40.8	3.10 ± 0.6	6.28 ± 1.20
D	3% Chitosan (>85% DD)	0.015	46.2	4.29 ± 1.40	5.48 ± 1.70

* statistically 95% confidential level

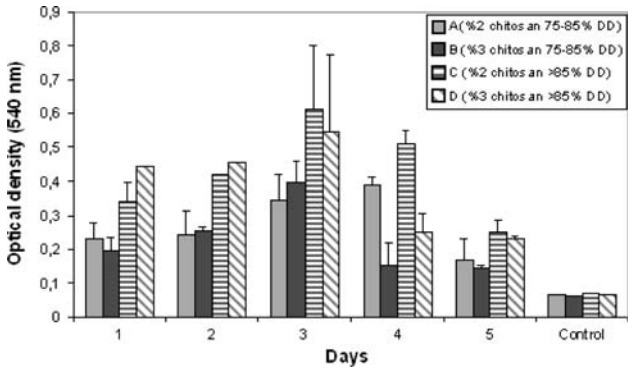

Fig. 5 Proliferation trends of L929 fibroblasts on chitosan scaffolds by MTT measurements

24 h incubation period, fibroblasts start to proliferate on scaffolds throughout the attached cells. The results showed an increase in cell number for all scaffolds by the end of 3 days of culture period. However, a significant increase of cell number was observed on >85% deacetylated chitosan scaffolds, may be due to the high proliferation trend when compared with low deacetylated chitosan scaffolds. After 3 days of culture period, a reduction in cell numbers were observed since the degree of cell viability is correlated with the magnitude of a growth response. Figure 5 shows the sharp decrease of cell number, obtained for chitosan scaffolds with 3% (w/v) composition by the end of four days of incubation period. Since the pore size of chitosan scaffolds of 3% (w/v) composition were relatively smaller, the observed sharp reduction of viability was probably due to the diffusion limitations between medium and cultured scaffold. In order to quantify the fibroblastic cell proliferation on chitosan scaffolds, specific growth rates (μ) were determined by Eq. 3. μ values were given in Table 3 together with doubling times and growth index values. The results indicate that cell growth rate, which is a measure of proliferation, was significantly higher on >85% deacetylated chitosan scaffolds. Comparisons demonstrate that >85% deacetylated chitosan scaffolds were more favorable for cell culturing.

Fluorescence imaging for cell viability

Cell infiltration and proliferation are crucial for a scaffold to support and guide tissue regeneration. Figure 6 represents the fluorescence microscope images of L929 fibroblasts cultured after 2 and 4 days for >85% deacetylated chitosan scaffolds. After 2 days of incubation period, a great number of fibroblasts started to proliferate on deacetylated chitosan scaffolds with 2% (w/v) composition (Fig. 6a). At the end of 4 days, it is clearly seen that cells were proliferated completely throughout the scaffold and were almost covering the surface and pores (Fig. 6b). This result, which is in good correlation with quantitative results of MTT assay, demonstrates that the chitosan scaffold preserves good cytocompatibility.

Cell morphology on chitosan scaffolds

The cell morphology on >85% deacetylated chitosan scaffolds for the second and fourth days of culture are given in Figs. 7 and 8, respectively. The images taken from CLSM at the second day of culture show that the cells adhered to the pores of scaffolds, spreaded and proliferated on the scaffolds (Fig. 7a, b). The SEM images on the fourth day of culture demonstrate that adhered cells started to spread and changed their spheroidal shape into ellipsoid-like shape (Fig. 8). Additionally, proliferated cells took the lamellar appearance, almost covering the surface of porous scaffold.

Conclusion

Four types of chitosan scaffolds with two different compositions and DDs, were investigated for their possible use in tissue engineering. This study has demonstrated that DD has significant influence on morphological structure and degradation characteristics of chitosan scaffolds. Chitosan scaffolds with higher DDs were morphologically observed to have higher interconnectivity and lower degradation rates. However, mechanical properties were more affected

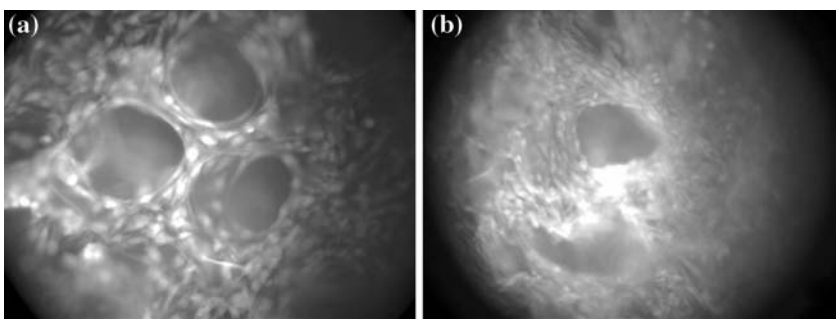

Fig. 6 Fluorescence microscope images of L929 fibroblasts cultured on 2% (w/v) >85% DD scaffolds (**a**) Second days of culture; (**b**) Fourth days of culture (magnification ×20)

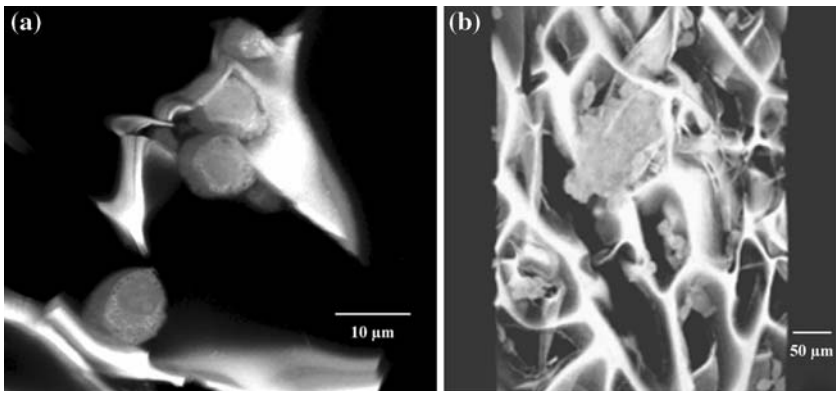

Fig. 7 Morphological structure of fibroblasts cultured on >85% deacetylated chitosan scaffolds; CLSM images at the second days of culture

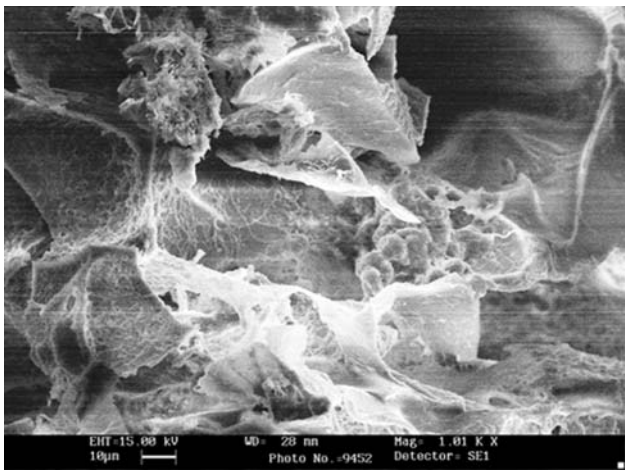

Fig. 8 Morphological structure of fibroblasts cultured on >85% deacetylated chitosan scaffold; SEM image, 4 days after incubation

by compositions of chitosan scaffolds rather than DDs. Here, we reported the effect of DD of chitosan scaffolds on fibroblastic cell proliferation as it has not been reported yet. Fibroblastic cell proliferation was found to have higher trend in high DD chitosan scaffolds when compared with lower DD chitosans. However, scaffolds of lower DD did not inhibit cell attachment and proliferation in contrary to the studies which have been done with chitosan films. This result may be attributed to the 3D structure of chitosan scaffolds. It is concluded that DD played a key role in cell adhesion as well as cell proliferation. Consequently, chitosan scaffolds with high DD can be considered as a potential candidate not only for cell cultivation but also for superficial soft tissue engineering applications.

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