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# Effect of collagen II coating on mesenchymal stem cell adhesion on chitosan and on reacetylated chitosan fibrous scaffolds

**Guillaume R. Ragetly · Dominique J. Griffon · Hae-Beom Lee · Yong Sik Chung**

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**Abstract** The biocompatibility and biomimetic properties of chitosan make it attractive for tissue engineering but its use is limited by its cell adhesion properties. Our objectives were to produce and characterize chitosan and reacetylated-chitosan fibrous scaffolds coated with type II collagen and to evaluate the effect of these chemical modifications on mesenchymal stem cell (MSC) adhesion. Chitosan and reacetylated-chitosan scaffolds obtained by a wet spinning method were coated with type II collagen. Scaffolds were characterized prior to seeding with MSCs. The constructs were analyzed for cell binding kinetics, numbers, distribution and viability. Cell attachment and distribution were improved on chitosan coated with type II collagen. MSCs adhered less to reacetylated-chitosan and collagen coating did not improve MSCs attachment on those scaffolds. These findings are promising and encourage the evaluation of the differentiation of MSCs in collagen-coated chitosan scaffolds. However, the decreased cell adhesion on reacetylated chitosan scaffold seems difficult to overcome and will limit its use for tissue engineering.

G. R. Ragetly (✉) · D. J. Griffon
Department of Veterinary Clinical Medicine, University of Illinois, Urbana, IL 61802, USA
e-mail: ragetly2@illinois.edu

H.-B. Lee
Department of Veterinary Medicine, Chonbuk National University, Jeonju, Republic of Korea

Y. S. Chung
Department of Textile Engineering, Chonbuk National University, Jeonju, Republic of Korea

## 1 Introduction

Tissue engineering is a rapidly developing field offering new perspectives in the treatment of damaged or diseased tissues. The basic premise of tissue engineering relies on the use of scaffolds to encourage cells to proliferate and organize their extracellular matrix (ECM) in order to form ex vivo a clinically functional tissue, exhibiting histochemical, biochemical and biomechanical properties identical to native, healthy tissue. Three key constituents form the basis of a tissue engineering approach, namely, cells, a scaffold, and signaling molecules [1]. The use of mesenchymal stem cells (MSCs) as a cell source is attractive because they can be harvested with less morbidity from a patient than most differentiated cells, [2–4] have self-renewal abilities, and can differentiate into several cell types [4, 5].

The cell behavior will be affected by the chemical composition and structural characteristics of the three-dimensional (3-D) scaffold, which will ultimately determine the performance of the tissue-engineered construct [1, 6–8]. The biocompatibility of biomaterials derived from chitin and their similarity to the glycosaminoglycans (GAG) naturally present in extracellular matrix make them particularly attractive as a candidate for skeletal tissue engineering [9]. Chitosan is the second most abundant natural polysaccharide, primarily obtained as a subproduct of shellfish by deacetylation of chitin [10]. Chitin and chitosan have been studied for use in a number of biomedical applications including wound dressings, drug delivery systems, and space filling implants and has gained FDA approval for human use [11, 12]. These biomaterials can be molded in various geometries and forms including fibrous scaffolds which have biomimetic properties [13–16]. Considerable attention has recently focused on chitosan and its

applications in the field of skeletal tissue engineering including cartilage, intervertebral disk, and bone [11, 13–20]. We have previously reported improved chondrogenesis but limited adhesion of chondrocytes and MSCs on chitosan sponges and meshes [15, 16, 21]. Others have confirmed these results, limiting the application of chitosan and chitin for tissue engineering [22–24]. The initial attachment of cells to a scaffold is a prerequisite for a successful tissue engineering outcome as it is the clincher of cell–matrix interactions [25]. Improving cell attachment and cell distribution on chitosan-derived scaffolds is therefore essential for in vitro cultivation of clinically relevant constructs.

The strategies most commonly employed to improve cell adhesion on biomaterials focus on reduction of non-specific protein adsorption by molecular modification of the biomaterial or immobilization of adhesion molecules to ensure controlled interaction between the cells and the scaffold [26]. The effect of specific molecules on cell adhesion is dependent on the chemical modification, the bulk biomaterial, and the type of cells evaluated [27]. The peptide Arg-Gly-Asp (RGD) is the coating agent most often used to promote the attachment of cells on material surfaces [28, 29]. However, RGD was found to inhibit fibroblast adhesion and proliferation on reacetylated chitosan films [26]. Moreover, the immobilization of synthetic RGD affects the ability of MSCs to differentiate [30]. Type II collagen is another candidate which could improve cell attachment via the interactions with the integrins $\alpha1$, $\alpha2$, $\alpha10$, and/or $\alpha11\beta1$ as well as the discoidin-domain receptors, and the annexin V receptor [31–34]. Type II collagen has been less commonly used for tissue engineering applications than type I collagen. Although type I collagen coating has been found to improve cell adhesion on chitosan, the use of type II collagen is more attractive as a biomimetic strategy to improve both MSCs adhesion and chondrogenic or osteogenic differentiation [35, 36]. Type II collagen differs from type I collagen in structure, distribution, and effects on cells [37, 38]. Type I collagen can be found in skeletal tissue but also in skin, cornea, arteries, internal organs, and granulation tissues whereas the natural distribution of type II collagen is more specific to skeletal tissues (hyaline cartilage, bone growth plate). In one study, chondrocyte proliferation and distribution were improved in type II collagen matrices compared to type I collagen matrices [39]. Type II collagen as a bulk biomaterial has been used in few tissue engineering applications [39–43]. Scaffolds composed of bulk type II collagen-poly-caprolactone improved chondrocyte adhesion and proliferation compared to poly-caprolactone scaffolds [41]. Although the mechanical properties and dimensional stability of type II collagen prevent its use as a bulk biomaterial, they do not affect its potential as a coating molecule for scaffolds used in skeletal tissue engineering [42]. To our knowledge, the evaluation of type II collagen coating has only been limited to one study on poly-lactide-coglycolide scaffolds [42]. The effect of type II collagen coating on chitosan and reacetylated chitosan scaffolds has not been evaluated.

The objectives of this study were to determine the extent to which reacetylation of chitosan and/or type II collagen coating could improve MSCs seeding. We hypothesized that MSCs adhesion will be greater on chitosan meshes compared to reacetylated chitosan meshes and that type II collagen coating will improve cell seeding efficiency on both chitosan and reacetylated chitosan scaffolds.

## 2 Materials and methods

### 2.1 Scaffolds

The chitosan (Texanmedtecho, Korea) used in this study has a molecular weight of 480 kDA, a degree of deacetylation of 92% and a viscosity of 370 cP at a 0.5% concentration. Chitosan powder was dissolved and stirred at room temperature for 48 h in 2 wt% aqueous acetic acid solution to obtain a 4 wt% chitosan solution.

Chitosan fibrous scaffolds were prepared using a wet spinning method previously reported. The chitosan solution was pumped into a 0.1 mm × 1,500 holes spinneret using the geared metering pump. To solidify the chitosan solution, the spinneret was immersed in an aqueous coagulation bath containing 10% sodium hydroxide. After exiting the coagulation bath, fibers were washed in alternative hot and cold water baths. Fibers were cut by 51 mm and carded to obtain the chitosan fibers web. The web was bonded by passing through the water-jet chamber and dried. Chitosan fibrous scaffolds were cut to measure 4 mm in diameter and 1.5 mm in height (group 1).

A portion of the chitosan fibers was reacetylated by suspension in 150 ml of methanol and acetic anhydride (1 mol per glucosamine unit). The mixture was stirred at 40°C for 24 h. The treated fibers were washed several times with 100% ethanol, and air-dried. Fibers were cut by 51 mm and carded to obtain the reacetylated chitosan fibers web. The web was bonded by passing through the water-jet chamber and dried. Reacetylated chitosan fibrous scaffolds were cut to measure 4 mm in diameter and 1.5 mm in height (group 2).

Two types of type II collagen solutions were used to coat the scaffolds. Solution 1 was obtained by dissolving 10 mg of type II collagen (Calf type II collagen, Elastin products company, Owensville, Missouri, USA) in 10 ml of 0.4 mg/ml of acetic acid. The two types of scaffolds (chitosan and reacetylated chitosan) were placed in solution 1 for 1 h. The chitosan scaffolds dissolved in the collagen solution.

The reacetylated chitosan scaffolds were freeze-dried (group 3). Solution 2 was obtained by adding ethanol to solution 1 at a concentration of 3 mg/ml to prevent dissolution of chitosan scaffolds. The two types of scaffolds (reacetylated chitosan (group 4) and chitosan (group 5)) were kept in solution 2 for 1 h and dried at room temperature.

A total of 30 scaffolds were prepared for each of the following groups:

1. chitosan scaffolds
2. collagen-coated chitosan scaffolds
3. reacetylated chitosan scaffolds
4. collagen-coated reacetylated chitosan scaffolds (without ethanol)
5. collagen-coated reacetylated chitosan scaffolds (with ethanol).

All scaffolds had a dry weight of 1.98 ± 0.18 mg. They were sterilized with ethylene oxide gas and rehydrated through a series of ethanol/phosphate buffered saline (PBS) solutions (100, 95, 75, 50, 0% ethanol) [44]. The scaffolds were subsequently incubated at 37°C on a shaker incubator in Dulbecco's modified Eagle's medium (DMEM, ATCC, Manassas, VA, USA) for 2 h prior to cell seeding [16].

## 2.2 Mesenchymal stem cells

A mesenchymal cell line (D1 ORL UVA, ATCC) derived from a multipotent mouse bone marrow stromal precursor was used for this study. Complete culture medium consisted of DMEM containing 4.5 g/l glucose (ATCC), supplemented with 10% fetal bovine serum (ATCC), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma, St. Louis, MO). Cells were harvested at their fourth passage and suspended in medium prior to seeding. A cell suspension containing $1.0 \times 10^6$ cells in 20 μl of medium was dropped on the surface of each scaffold (15 scaffolds per group, 5 groups) and placed in untreated six-well non-tissue culture plates. Each construct was allowed to incubate at 37°C for 1 h. After 1 h, 4 ml of culture medium was added to each well and the plates were placed on a platform shaker oscillating starting at 30 rpm and increased to 60 rpm after 3 h. The constructs were maintained at 37°C and 5% $CO_2$ in culture medium for 24, 48, or 72 h.

## 2.3 Evaluation of the scaffolds

### *2.3.1 Fourier-transform infrared spectroscopy*

Fourier-transform infrared (FTIR) spectra of each scaffold type (n = 3) were analyzed by spectrophotometry (Nicolet Nexus FTIR 670, Thermo Electron, Waltham, MA, USA) of dehydrated specimens ground with KBr powder and compressed into pellets. Element analysis was used to determine the degree of substitution of the *N*-acyl groups and the presence of collagen.

### *2.3.2 Scanning electron microscopy*

Each scaffold (n = 2 per group) was mounted and sputter coated with gold–palladium prior to examination with scanning electron microscopy (SEM, Hitachi S4700, Schaumburg, IL, USA) at 1.0 kV. Criteria evaluated included fiber size, surface characteristics, and the presence of type II collagen.

### *2.3.3 Transmission electron microscopy*

The scaffolds (n = 2 per group) were fixed in a Karnovsky's Fixative in phosphate buffered 2% Glutaraldehyde and 2.5% Paraformaldehyde. Microwave fixation was used with this primary fixative and the scaffolds were then washed in Sorenson's Phosphate buffer with no further additives. Microwave fixation was also used with the secondary 2% Osmium Tetroxide fixative, followed by the addition of 3% Potassium Ferricyanide for 5 min. After washing with water, saturated Uranyl Acetate was added for 1 h for en bloc staining. The tissue was dehydrated in a series of increasing concentrations of ethanol. Acetonitrile was used as the transition fluid between ethanol and the epoxy. Infiltration series were done with an epoxy mixture using the epon substitute Lx112. The resulting blocks were polymerized at 90°C overnight and trimmed. Transverse (n = 3) and longitudinal (n = 3) sections of 0.35 μm were cut with diamond knives in each specimen. Light microscopy slides were made with one transverse and one longitudinal section and stained with Toluidine Blue O and Basic Fucshine. Transmission electron microscopy (TEM) sections (two transverse and two longitudinal sections) were stained with Uranyl Acetate and Lead Citrate, and examined with a Transmission Electron Microscope (Hitachi H600, Schaumburg, IL, USA). Criteria evaluated included homogeneity of the fiber size, presence of a coating at their surface and homogeneity of the coating.

### *2.3.4 Type II collagen content*

Type II collagen content per scaffold (n = 3 per group) was determined by an enzyme linked immunosorbent assay (Arthrogen-CIA® Native Type II Collagen Detection Kit, Chondrex, Redmond, WA) after digestion of the scaffold in pepsin and elastase [16]. Collagen fibrils were solubilized with pepsin and further digested with pancreatic elastase. The optical density of the reacted collagen with monoclonal antibody was read at 490 nm [16].

#### 2.3.5 Water content and porosity

Scaffolds (n = 5 per group) were weighed before and after dehydration to determine their wet and dry weights, respectively. The water content was subsequently calculated as:

$$\text{Water content}(\%) = \left(\text{Weight}_{\text{wet}} - \text{Weight}_{\text{dry}}\right)/\text{Weight}_{\text{wet}}$$

The porosity was calculated using the equation: [41]

$$\text{Porosity}(\%) = \left[\left(\text{Weight}_{\text{wet}} - \text{Weight}_{\text{dry}}\right)/\text{Water density}\right]/\text{Sample volume}$$

The sample volume was 0.03 $cm^3$ for all scaffolds. The water density was considered 1 $g/cm^3$.

#### 2.3.6 Mechanical property measurement of the chitosan and reacetylated chitosan scaffolds

The tensile properties of the scaffolds were evaluated according to ASTM D-5035 using universal instron tester (Model LR5kPlus, Lloyd instrument Co.). The chitosan and reacetylated chitosan scaffolds were cut to 100 × 25 mm. Five specimens were tested for each groups. A 5 N load cell was used at a constant rate of 100 mm/min. The same tests were repeated after the scaffolds were placed in PBS buffer solution at 37°C for 1, 2, and 3 weeks. The breaking strength was measured to evaluate the change of bulk properties and mechanical stability.

### 2.4 Evaluation of constructs

#### 2.4.1 Evaluation of cell binding kinetics and cell viability

Cell binding kinetics and viability were evaluated after the initial incubation phase, once fresh media was added to each well. The number of cells suspended in medium and their viability were evaluated at 1, 2, 4, 6, 18 and 22 h after the initial seeding via trypan blue exclusion. Measurements were taken separately for each scaffold type among two wells. The cell concentration in the medium was compared for each time point [45].

#### 2.4.2 Live/dead assay

The viability of cells in the constructs 48 h after seeding was determined using the Live/Dead Viability/Cytotoxicity Kit (Molecular Probes, Carlsbad, CA, USA) according to the manufacturer's protocol. Constructs (n = 2 per group) were washed three times in sterile PBS for 2 min, placed on a glass slide, and immersed in 200 µl of PBS solution containing 2 mM calcein AM and 4 mM ethidium homodimer 1 prior to incubation for 40 min at room temperature. Confocal microscopy (Olympus BX50 Confocal Microscope, Olympus, Center Valley, PA, USA), using Melles Griot Argon and Krypton lasers at excitation wavelengths of 488 and 568 nm, allowed the visualization of calcein AM (labeling live cells = green fluorescence) and ethidium homodimer-1 (labeling dead cells = red fluorescence). The intensities of viable and dead cells were recorded on four field of view at a magnification of 40×. The slides were analyzed using a specific software (Fluoview, Olympus) to determine the percentage of viable cells.

#### 2.4.3 Weights, water, and DNA content

Constructs were weighed 24 (n = 5 per group) and 72 (n = 5 per group) hours after seeding before and after dehydration to determine their wet and dry weights, respectively. The water content was subsequently calculated. After dehydration, the constructs were assayed for their DNA contents. Samples were digested in papain (Sigma Inc., St-Louis, MO, USA) for 16 h at 60°C. A fluorometric assay with Hoechst 33258 was used to evaluate DNA content [46]. The cell seeding efficiency was calculated as a ratio between the numbers of cells contained in constructs after 24 h of culture compared to the number of cells initially seeded on each scaffold. The number of cells contained in the constructs was calculated using the average DNA content per cell (5.34 pg per cell) as evaluated by fluorometric assay at the time of cell seeding on cell suspensions containing $1 \times 10^6$ and $2 \times 10^6$ cells [16].

The number of cells present in the well unattached to a scaffold was evaluated by DNA quantification 24 (n = 2 per group) and 72 (n = 2 per group) hours after seeding. The cell-media solutions were collected and centrifuged at 250 rpm/rcf for 10 min and the pellet was assayed for DNA content using the above protocol.

#### 2.4.4 Transmission electron microscopy

The evaluation protocol used for the constructs was similar to that described above for the scaffolds. One construct per group was evaluated at 24, 48, and 72 h after seeding. Criteria evaluated via TEM included cell morphology, cell size, cell attachment to the support, and presence of cytoplasmic extensions.

#### 2.4.5 Histology

Light microscopy slides were made following the same protocol as described above for TEM evaluation of the scaffolds. Histological sections were stained with

Toluidine Blue O and Basic Fucshine and evaluated to assess the cell distribution.

The constructs (n = 2 per group at 72 h) were fixed in 10% neutral buffered formalin, embedded in plastic and cut via microtome to produce three 8 μm-thick sections (one superficial, one in the middle, and one at the bottom) [47]. Sections were stained with a trichrome stain. Slides were examined for cell morphology, cell distribution within slides and between slides of the same construct, and integrity of the scaffold.

#### 2.4.6 Scanning electron microscopy

Constructs were fixed in a 2.5% gluteraldehyde solution in a sodium cacodylate buffer for 2 h. After rinsing with the sodium cacodylate buffer, they were submerged in 1% osmium tetroxide in 0.1 M sodium cacodylate for 90 min. Following a buffer rinse, the constructs were dehydrated through an ethanol series. Finally, constructs were placed in hexamethyldisilazane for 45 min and left under a fume hood until completely dry. A total of two sections per construct (surface and bottom, n = 2 per group at 72 h) were mounted for each construct and sputter coated with gold–palladium prior to examination with SEM at 1.0 kV. Criteria evaluated included cell attachment to the support, presence of cytoplasmic extensions, cell density, and integrity of the scaffold.

### 2.5 Statistical analysis

Dry and wet weights, increase in dry weight, water contents, cell counts in medium, cell viability, DNA content, and increase in DNA content between 24 and 72 h were compared between the five groups with a risk factor of less than 0.05 considered statistically significant. All data were expressed as mean ± standard deviation. Statistical differences were evaluated between the groups with an ANOVA using Systat 11.0 statistical software (Wilkinson). A t-test was performed to compare the mechanical properties of the chitosan and reacetylated chitosan scaffolds. Post-hoc analyses were performed with the LSD test.

## 3 Results

### 3.1 Evaluation of the scaffolds

#### 3.1.1 FTIR

The degree of acetylation (DA) was 8% for the chitosan scaffolds and 96% for the reacetylated chitosan scaffolds. The presence of collagen was confirmed by the presence of bands typical of amide I (1658 $cm^{-1}$), amide II (1552 $cm^{-1}$), and amide III (1240 $cm^{-1}$) (Fig. 1) [48].

#### 3.1.2 Scanning electron microscopy

All fibers had a diameter of 13–15 μm. They appeared homogeneous within scaffolds and among the different groups. The difference between chitosan and collagen was difficult to distinguish because of similar contrast with SEM evaluation.

#### 3.1.3 Transmission electron microscopy

All fibers appeared of similar size among all TEM samples. A layer of collagen was observed at the surface of the fibers in all collagen-coated scaffolds (Fig. 2). No difference in coating was observed between the center and the periphery or between scaffolds. Aggregation of collagen molecules was observed occasionally in the center as well as in the periphery and in all types of collagen-coated scaffolds (Fig. 2).

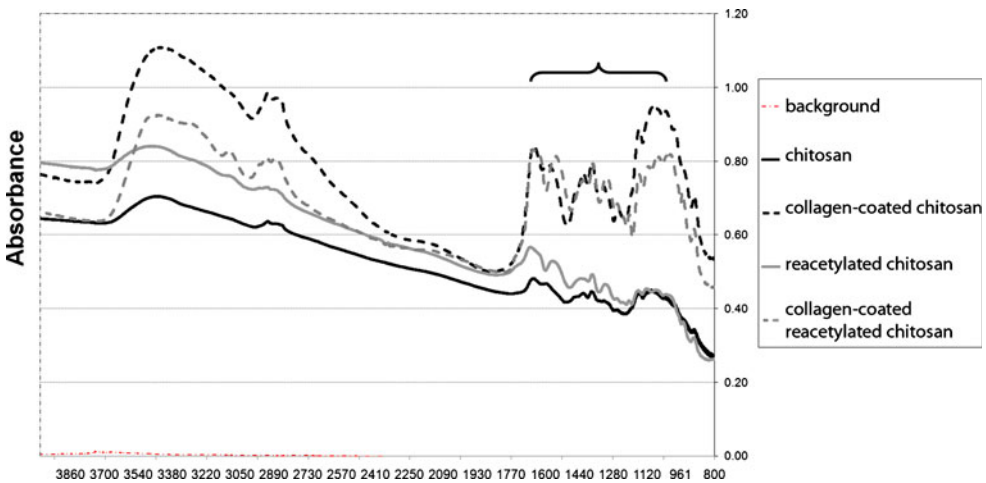


**Fig. 1** Fourier transform infrared spectroscopy results. The element analysis was used to determine the degree of substitution of the *N*-acyl groups. The presence of collagen was confirmed in the coated scaffolds by the presence of bands typical of amide I, II, and III (brace)

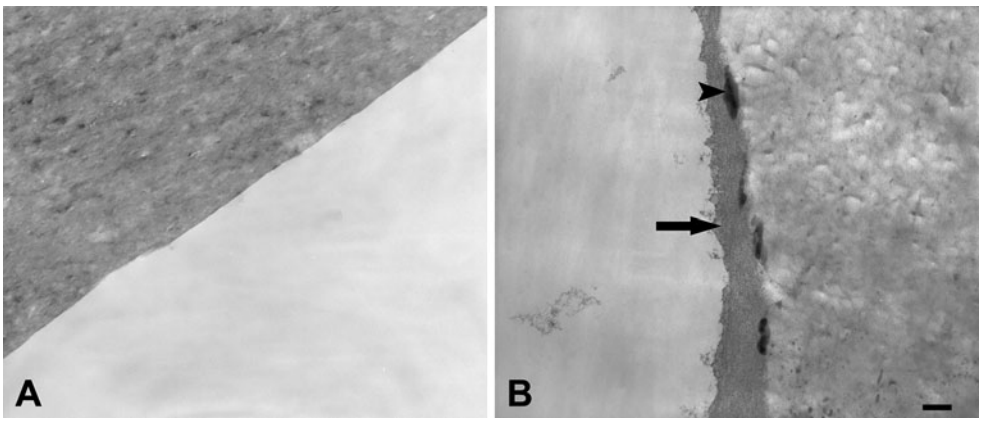


**Fig. 2** Transmission electron microscopy of the edge of a fiber from a chitosan scaffold (**A**) and a collagen-coated chitosan scaffold (**B**). The *black arrow* indicates the collagen layer seen at the surface of the collagen-coated chitosan fiber; the *arrowhead* indicates an aggregation of collagen molecules (magnification x50000). *Scale bar*: 0.1 μm

### *3.1.4 Type II collagen content*

Type II collagen was not detected in the non-coated scaffolds. The type II collagen content was 4.11 ± 1.39 μg/mg for the collagen-coated chitosan scaffolds, 4.98 ± 0.15 μg/mg for the collagen-coated reacetylated chitosan scaffolds (without ethanol), and 8.04 ± 2.00 μg/mg for the collagen-coated reacetylated chitosan scaffolds (with ethanol). The collagen-coated reacetylated chitosan scaffolds contained more type II collagen than the collagen-coated chitosan and the collagen-coated reacetylated chitosan (without ethanol) scaffolds ($P = 0.032$ and 0.044, respectively).

### *3.1.5 Water content and porosity*

No difference was found in water content nor porosity among groups (mean values among the different groups of 92.5 ± 1.8%, $P = 0.653$ and 86.4 ± 20.7%, $P = 0.524$, respectively) (Table 1).

### *3.1.6 Mechanical property measurement of the chitosan and reacetylated chitosan scaffolds*

No difference in stress, strain and maximum load to failure was found between the chitosan and reacetylated chitosan scaffolds (Table 2).

**Table 1** Summary statistics of the scaffold water content and porosity, the construct water content, and the cell seeding efficiency of the scaffolds or constructs

| Composition | Chitosan | Collagen-coated chitosan (ethanol) | Reacetylated chitosan | Collagen-coated reacetylated chitosan | Collagen-coated reacetylated chitosan (ethanol) |
|---|---|---|---|---|---|
| Scaffold water content (%) | 92.9 ± 1.7 **A** | 92.7 ± 1.8 **A** | 92.7 ± 1.4 **A** | 92.5 ± 1.1 **A** | 92.5 ± 1.8 **A** |
| Scaffold porosity (%) | 87.2 ± 19.0 **A** | 91.8 ± 22.6 **A** | 93.7 ± 20.0 **A** | 84.1 ± 19.8 **A** | 85.1 ± 20.6 **A** |
| Construct water content (%) | 90.0 ± 2.0 **A** | 91.9 ± 3.0 **A** | 91.4 ± 2.4 **A** | 90.3 ± 1.2 **A** | 90.4 ± 3.9 **A** |
| Cell seeding efficiency (%) | 47.2 ± 8.6 **B** | 81.8 ± 32.5 **A** | 25.5 ± 10.7 **C** | 24.6 ± 3.6 **C** | 23.5 ± 6.4 **C** |

A, B, C: groups with different letters differ statistically ($P < 0.05$)

**Table 2** Mechanical properties and stability of the chitosan and reacetylated chitosan scaffolds

| | | Week 0 | Week 1 | Week 2 | Week 3 |
|---|---|---|---|---|---|
| Chitosan scaffold | Maximum load (N) | 5.27 | 5.24 | 5.13 | 5.24 |
| | Stress (N/mm) | 0.410 | 0.407 | 0.394 | 0.402 |
| | Strain (%) | 48.6 | 50.5 | 38.2 | 51.6 |
| Reacetylated chitosan scaffold | Maximum load (N) | 5.30 | 5.21 | 5.12 | 5.06 |
| | Stress (N/mm) | 0.415 | 0.409 | 0.398 | 0.395 |
| | Strain (%) | 34.8 | 35.6 | 35.8 | 21.6 |

### 3.2 Evaluation of constructs

#### *3.2.1 Evaluation of cell binding kinetics and cell viability*

Cell death in the medium was negligible (less than 5%) throughout the experiment for all seeding techniques as assessed by trypan blue exclusion. Less than 10% of the cells remained in suspension 1 h after the seeding for all scaffold types. No difference in cell concentration was found until 6 h after seeding. More cells remained in suspension in the wells of the chitosan scaffolds than in other groups 6 h after seeding ($P = 0.014$) but this difference was not found at any later time points ($P > 0.497$).

#### *3.2.2 Live/dead assay*

The viability of the MSCs estimated by a live/dead fluorescent assay 48 h after seeding did not differ among the five groups and remained above 83.6% for all constructs (93.9% ± 8.9, $P = 0.447$).

#### *3.2.3 Weights, water, and DNA content*

No difference was found between groups in wet weight, dry weight and water content after seeding ($P = 0.249$, 0.403, and 0.432, respectively). The water content was 90.8 ± 2.6% (Table 1).

The DNA remained stable within groups from 24 to 72 h after seeding. ($P > 0.172$ for all scaffold types). The average DNA content was 2.51 ± 0.46 μg for the chitosan constructs, 4.37 ± 1.73 μg for the collagen-coated chitosan constructs, 1.36 ± 0.57 μg for the reacetylated chitosan constructs, 1.32 ± 0.19 μg for the collagen-coated reacetylated chitosan constructs (without ethanol), and 1.25 ± 0.34 μg for the collagen-coated reacetylated chitosan constructs (with ethanol) (Fig. 3). The collagen-coated chitosan constructs contained more DNA than any other constructs at 24 and 72 h ($P < 0.001$ for all comparisons). Chitosan constructs contained more DNA than the collagen-coated reacetylated chitosan constructs at 24 h ($P = 0.030$ and 0.045) and than all reacetylated chitosan constructs at 72 h ($P = 0.002$–$0.004$). No difference was found between the reacetylated chitosan constructs at either 24 or 72 h ($P > 0.696$ and $P > 0.856$, respectively). The cell seeding efficiency after 24 h of culture was 47.2 ± 8.6% for the chitosan constructs, 81.8 ± 32.5% for the collagen-coated chitosan constructs, 25.5 ± 10.7% for the reacetylated chitosan constructs, 24.6 ± 3.6% for the collagen-coated reacetylated chitosan constructs (without ethanol), and 23.5 ± 6.4% for the collagen-coated reacetylated chitosan constructs (with ethanol) (Table 1). The efficiency of cell seeding was greater on collagen-coated chitosan scaffolds than any other construct ($P < 0.001$ for all comparisons). Seeding was improved on chitosan scaffolds compared to all reacetylated chitosan constructs ($P = 0.016$–$0.034$). No difference was detected among the different reacetylated chitosan constructs ($P > 0.9$).

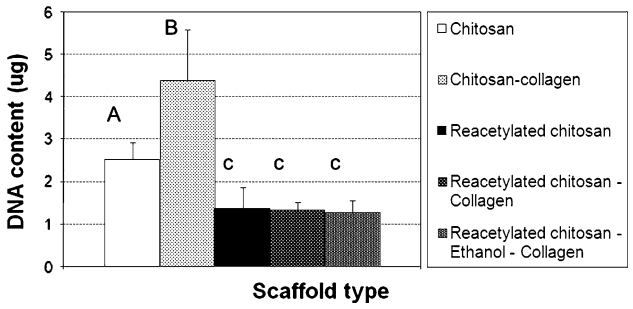


**Fig. 3** Average DNA content of the constructs 24 and 72 h after seeding. *A*, *B*, *C* groups with different letters differ statistically ($P < 0.05$)

No difference in the DNA content of the well was found after 24 h ($P = 0.588$). However, the DNA content of the wells of chitosan constructs was greater than the one of the wells of all other constructs at 72 h ($P = 0.014$).

#### *3.2.4 Transmission electron microscopy*

Cells exhibited similar morphological features typical of mesenchymal stem cells including a spindle-shape and elliptical nucleus with usually multiple nucleoli, various mitochondrial profiles, and small vacuoles. No difference in cell morphology was observed between the constructs at 24, 48, and 72 h after seeding. No difference in cell attachment could be observed between the different groups.

#### *3.2.5 Histology*

The cell density and distribution among each construct was assessed with the histological sections stained with Toluidine Blue O and Basic Fucshine. Findings were in agreement with those of DNA content. The cell distribution also appeared to differ among groups (Fig. 4). Cells in chitosan constructs tended to be grouped rather than uniformly distributed, whereas cell distribution seemed more uniform in collagen-coated chitosan constructs. Although less obvious, a similar trend was observed in reacetylated chitosan constructs where cells appeared more uniformly distributed along collagen-coated fibers. Examination of the samples stained with trichrome confirmed the findings obtained with the light microscopy slides of the TEM in terms of subjective cell numbers and distribution among groups. Cells exhibited the appearance of MSCs in all constructs (Fig. 5). The chitosan structure appeared intact in all constructs.

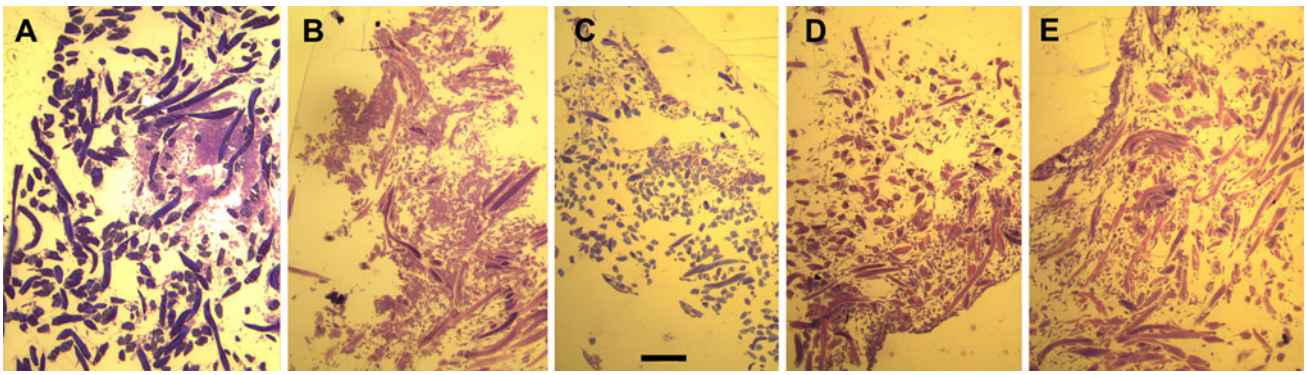


**Fig. 4** Histological analysis with toluidine blue O and basic fucshine 24 h after seeding allowing evaluation of cell distribution within each construct. **A** Chitosan constructs. **B** Collagen-coated chitosan constructs. **C** Reacetylated chitosan constructs. **D** Collagen-coated reacetylated chitosan constructs (without ethanol). **E** Collagen-coated reacetylated chitosan constructs (with ethanol) (magnification ×10, bar 100 μm)

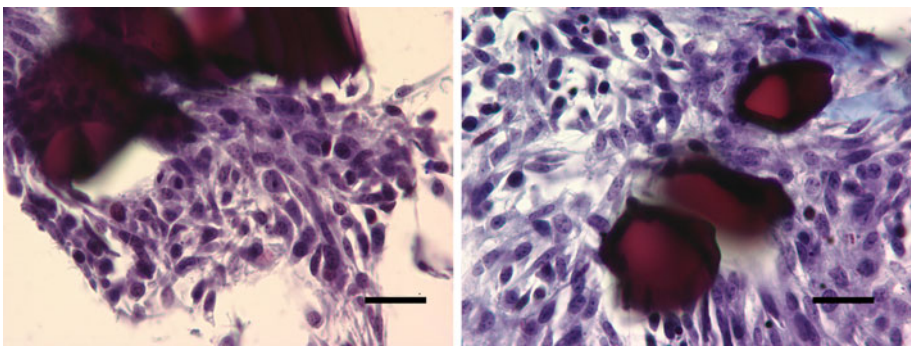

**Fig. 5** Histological analysis with trichrome stain 72 h after seeding of a chitosan construct (*left*) and a collagen-coated chitosan construct (*right*). Cells exhibited the appearance of MSCs and were well attached to the scaffold (magnification ×100, *bar* 30 μm)

### *3.2.6 Scanning electron microscopy*

Most MSCs exhibited a spindle-shape with prominent cytoplasmic extensions. No major differences in cell morphology were observed among groups. The cells tended to be uniformly distributed within each evaluated surface (surface or bottom). However, marked differences in cell density were observed and were in agreement with those of DNA content (Fig. 6). The reacetylated chitosan constructs contained fewer cells at the surface and at the bottom than the chitosan-based constructs. More cells were present at the surface and at the bottom of collagen coated chitosan constructs than in all the other groups. It was not possible to count the cells because they exhibited a spindle-shape with prominent cytoplasmic extensions and tended to form sheets [15, 16]. Finally, the structural integrity of fibers seemed intact in all groups.

## 4 Discussion

The initial attachment of cells during seeding is a pre-requisite for a successful tissue engineering outcome as it is the clincher of cell–matrix and cell–cell interactions [25]. Modifying chitosan scaffolds to optimize cell adhesion within the 3-D matrices is crucial to improve seeding yield and uniformity of cell distribution. The main findings of this study are that: (1) chitosan fibrous scaffolds can be chemically modified to alter their DA and/or their type II collagen coating, (2) type II collagen coating on chitosan fibrous scaffolds improves cell adhesion and cell distribution during seeding, and (3) increasing the degree of acetylation of chitosan scaffolds limits cell adhesion which cannot be effectively overcome by type II collagen coating.

Considerable attention has recently focused on chitosan scaffolds but their use has been limited because of their deficient properties for cell adhesion [22–24]. Our results confirm the low seeding efficiency of chitosan scaffolds (47.2%) seeded with MSCs. Histology, SEM and TEM evaluations correlated with the quantitative analysis. Cells seem to display greater affinity for each other than for the surface of chitosan fibers, forming clusters that contributed to the uneven cell distribution within constructs. The structure, wettability and porosity of scaffolds were consistent with previous reports [49]. The variability in porosity between the scaffolds may reflect scaffold variation

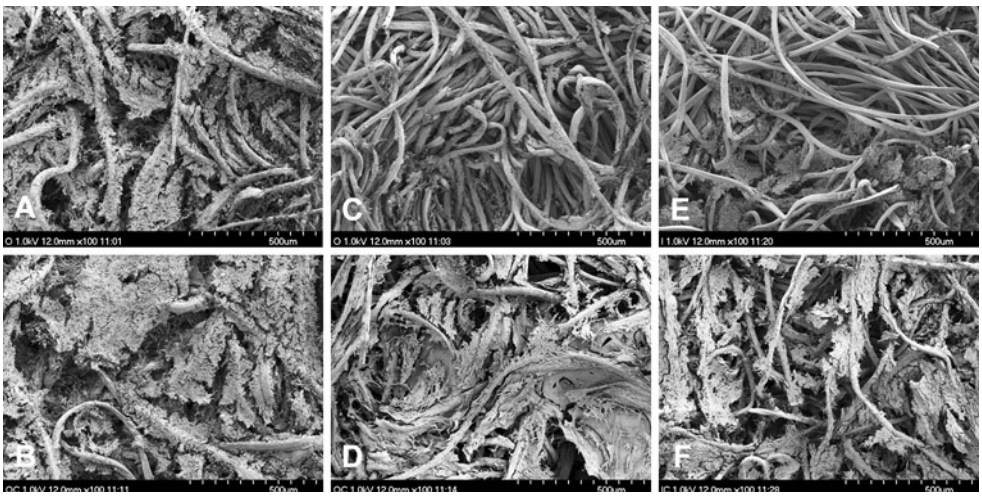


**Fig. 6** Scanning electron microscopy of the constructs 72 h after seeding with MSCs. **A** Surface of a chitosan construct. **B** Surface of a collagen-coated chitosan construct. **C** Bottom of a chitosan construct. **D** Bottom of a collagen-coated chitosan construct. **E** Surface of a reacetylated chitosan construct. **F** Surface of a collagen-coated reacetylated chitosan construct (magnification ×100)

or lack of precision of the measurement technique. The variation is more likely due to the technique since the scaffolds had the same structure on SEM evaluation and a low variability in their dry weight. This technique was used because the equation was found to be reliable to assess the porosity [41]. Coating chitosan fibers with type II collagen did not affect the structural properties of the scaffolds but improved cell adhesion. This structural similarity between scaffolds allows direct evaluation of the effect of chemical composition (collagen coating) on cell seeding [50, 51]. The coating did not impact cell viability but increased the cell seeding efficiency by 73%. The greater numbers of cells unattached to chitosan scaffolds in the medium (at 6 h) and in the well (at 72 h) compared to the collagen-coated chitosan scaffolds supports the effect of type II collagen on cell attachment. Type II collagen coating also improved cell distribution in collagen-coated constructs improving attachment to fibers rather than formation of clusters compared to chitosan constructs. These effects may be attributed to the presence binding sites in type II collagen, such as the amino acid sequences GFOGER and RGD. These binding sites have been found to promote cell attachment by the integrins $\alpha 1$, $\alpha 2$, $\alpha 10$, and/or $\alpha 11\beta 1$, the discoidin-domain receptors, and the annexin V receptor [31–34]. The adhesion of the cells to the matrix is believed to act as a clincher for intracellular signals, influencing subsequent cell–matrix interaction and cell differentiation [25, 32]. Type II collagen could therefore also improve the differentiation of the MSCs to form skeletal tissue. In fact, the synergistic effect of growth factors and extracellular type II collagen on Smad 2 phosphorylation has been shown to increase the level of ECM mRNA expression [52, 53]. Also, chondrogenic differentiation and endochondral ossification proceed through condensation. This formation of cell clusters due to increased cell density secondary to type II collagen coating can promote cadherin-mediated cell–cell interactions for skeletal tissue engineering. Future studies should be conducted to evaluate the chondrogenic and/or osteogenic differentiation of MSCs in the type II collagen coated chitosan scaffolds.

The influence of deacetylation of chitosan on cell attachment remains controversial and varies between reports and cell types [54]. In several studies, deacetylation improved the attachment of fibroblasts, Schwann cells, keratinocytes, and neurons to chitosan films or sponges [22–24, 55–57]. However, in others, a greater degree of acetylation of chitosan did not affect the attachment of chondrocytes, fibroblasts, or osteoblasts [58–61]. These conflicting results may be due to differences in origin, molecular weight, and/or viscosity of the agents tested (chitosan, chitin, re-acetylated chitosan) as well as differences in experimental design such as cell population and serum supplementation [60, 61]. We describe for the first time a decreased cell attachment of MSCs on reacetylated chitosan scaffolds (DA: 96%) compared to chitosan (DA: 8%). The scaffolds were produced from the same source of chitosan to avoid variation due to characteristics other than DA. Cells were seeded in medium containing serum, routinely recommended for culture of MSCs. The inverse relationship between adhesion of MSCs and the DA of chitosan scaffolds could be explained by several possible mechanisms. It was first suspected that the amine groups of the deacetylated form of chitosan would remain protonated to $-NH_3^+$ in media, resulting in a cationic nature primarily responsible for electrostatic interactions between protonated amine groups and negatively charged cell surfaces [55,

61]. However, the majority of the ammonium groups become dissociated and subsequently uncharged when the medium reaches a pH close to the pKA of chitosan (6.46–6.8) [22]. This explains why the zeta potentials of chitosan films were null or slightly positive in neutral medium and not affected by the DA [62]. The cell adhesion characteristics of the chitosan may therefore not be directly related to direct cell–matrix interactions but instead, correlate with the differential adsorption of proteins present in the culture medium to the biomaterial [63]. Although no correlation has yet been established between the adsorption of type II collagen and the DA of scaffolds, the mechanism of protein adsorption in media containing serum most likely differs from that of single protein adsorption in acidic conditions. Chitosan with a low DA may accelerate cell adhesion after forming polyelectrolyte complexes with serum components such as heparin, platelet-derived growth factor, laminin, or fibronectin [64, 65]. Chitosan with a lower DA can bind more growth factors in the serum and protect them from degradation and/or present them to the cells in an active form [65, 66]. This could potentially explain the absence of effect of the DA on fibroblast attachment and proliferation found by Hamilton et al. in serum free media [60]. The DA of chitosan scaffolds has been shown to impact the final constructs in skeletal tissue engineering applications. Several studies have reported improved characteristics of the extracellular matrix produced by chondrocytes, [58, 59] or osteoblasts [22, 61] when these cells were cultured on chitosan scaffolds of higher DA. These publications justify our attempts to overcome the poor cell adhesion on chitin and reacetylated chitosan [55, 56, 60]. Type II collagen did not improve the overall cell seeding efficiency of reacetylated chitosan constructs. Even if the cell distribution appeared more homogeneous in the collagen-coated constructs, the poor adhesion properties of reacetylated chitosan seem difficult to overcome.

## 5 Conclusions

Reacetylation of chitosan fibers was detrimental to the attachment and the distribution of MSCs, regardless of collagen coating. Cell adhesion on reacetylated chitosan scaffolds seems difficult to overcome and will likely limit its clinical application. Type II collagen coating improved MSCs adhesion and distribution on chitosan fibers but had no effect on reacetylated chitosan scaffolds. The seeding efficiency of 82% and the high kinetic rate of the collagen-coated chitosan scaffolds meet the criteria for application in tissue engineering. These findings are promising and encourage the evaluation of the differentiation of MSCs in chitosan fibrous scaffolds coated with type II collagen. However, future studies should evaluate the differentiation of MSCs in chitosan fibrous scaffolds coated with type II collagen to assess its biomimetic properties for cartilage and/or bone tissue engineering applications.

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