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Injectable chitosan-based hydrogels for cartilage tissue engineering

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ABSTRACT

Water-soluble chitosan derivatives, chitosan-*graft*-glycolic acid (GA) and phloretic acid (PA) (CH-GA/PA), were designed to obtain biodegradable injectable chitosan hydrogels through enzymatic crosslinking with horseradish peroxidase (HRP) and H_2O_2. CH-GA/PA polymers were synthesized by first conjugating glycolic acid (GA) to native chitosan to render the polymer soluble at pH 7.4, and subsequent modification with phloretic acid (PA). The CH-GA43/PA10 with a degree of substitution (DS, defined as the number of substituted NH_2 groups per 100 glucopyranose rings of chitosan) of GA of 43 and DS of PA of 10 showed a good solubility at pH values up to 10. Short gelation times (e.g. 10 s at a polymer concentration of 3 wt%), as recorded by the vial tilting method, were observed for the CH-GA43/PA10 hydrogels using HRP and H_2O_2. It was shown that these hydrogels can be readily degraded by lysozyme. *In vitro* culturing of chondrocytes in CH-GA43/PA10 hydrogels revealed that after 2 weeks the cells were viable and retained their round shape. These features indicate that CH-GA/PA hydrogels are promising as an artificial extracellular matrix for cartilage tissue engineering.

1. Introduction

Tissue engineering is a promising method for cartilage regeneration. In this approach, a scaffold as a temporary artificial extracellular matrix (ECM) is needed to accommodate cultured cells and guide their growth [1]. To allow cell survival in the artificial ECM, it is required to use scaffolds, which have properties resembling the native extracellular matrix such as a high water content and sufficient transport of nutrients and waste products. Because hydrogels, water-swollen networks of crosslinked hydrophilic polymers, are in general compatible with proteins, cells and surrounding tissues [2,3], they are regarded as highly suitable materials for artificial ECMs for tissue engineering.

Recently, injectable hydrogels that are in situ formed after injection at the defect site have received much attention in tissue engineering [4]. Injectable hydrogels have the advantage that implantation surgery can be replaced by a simple minimally invasive injection procedure. Moreover, the incorporation of cells and bioactive molecules like growth factors can be readily performed and the gels can be formed in any desired shape in good alignment with the surrounding tissue.

Chitosan has been widely investigated for biomedical applications such as controlled drug and protein delivery [5–7], non-viral gene delivery [8] and tissue engineering [9–12]. Chitosan is a polycationic polysaccharide comprising glucosamine and *N*-acetylglucosamine residues. Moreover, chitosan can be enzymatically degraded *in vivo* by lysozyme [13,14], a polycationic protein present in the ECM of human cartilage [15,16]. These features make chitosan a potential material for use in cartilage tissue engineering to modulate chondrocyte morphology, differentiation and stimulating chondrogenesis. *In vitro* studies demonstrated that chitosan-based matrices not only efficiently support chondrogenic activity [12,17], but also allow the expression of cartilage ECM proteins by chondrocytes [17]. Chondrocytes that are cultured in chitosan scaffolds may maintain their inherent round morphology [10]. Cui et al. showed that surface modification of PLLA films with chitosan leads to increased cell adhesion, proliferation, and correspondingly, improved glucosaminoglycan (GAG) production and collagen type II synthesis as compared to unmodified PLLA film [18]. A major drawback of chitosan is its poor solubility in neutral solutions. The chitosan backbone has to be derivatized with hydrophilic moieties to afford water-soluble chitosans [19].

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In the past years, many investigators have developed injectable chitosan-based hydrogels for cartilage repair [20–23]. Injectable chitosan hydrogels have been prepared by either physical or chemical crosslinking methods. The reversible physical interactions in poly(*N*-isopropyl-acrylamide) or PEG grafted chitosan derivatives have been used to prepare physically crosslinked hydrogels [5,20,22,24]. Such physical gels generally exhibit a low stability, low mechanical strength and fast degradation. In another approach, chemically crosslinked injectable chitosan hydrogels were prepared using redox-initiated crosslinking [21,25] and photo-initiated crosslinking [6,11,26,27]. Moreover, gelation time, gel modulus and hydrogel degradability of these chemically crosslinked hydrogels can be adjusted by the molecular weight of polymers and the crosslinking densities. For example Hong et al. prepared methacrylated chitosan-based hydrogels using ammonium persulfate and *N,N,N′,N′*-tetramethylethylenediamine and showed that by increasing the concentration of the initiator, the gelation time could be reduced and the enzymatic degradation of the resulting hydrogels decreased. However, a concomitant high cytotoxicity with low cell viability (<30%) at a high concentration of initiator was observed after a short cell culturing time of 4 days [21].

We previously reported on fast in-situ forming hydrogels from dextran–tyramine conjugates [28]. These hydrogels were formed within 1 min at optimal conditions upon enzymatic coupling of tyramine phenol moieties using horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2). Moreover, the hydrogels showed a high gel content and mechanical strength. In this study, we report on injectable biodegradable hydrogels based on chitosan derivatives using enzymatic crosslinking for cartilage regeneration. The gelation and degradation rates of these newly developed chitosan-based hydrogels as well as their mechanical properties were determined. Additionally, the *in vitro* cytocompatibility of the gels and the morphology of incorporated chondrocytes in time were studied.

2. Materials and methods

2.1. Materials

Chitosan (low molecular weight, viscosity = 20–200 cP, 1 wt% in 1 vol% acetic acid) was obtained from Aldrich. The degree of deacetylation (DD) of chitosan was estimated from ^{1}H NMR spectra as described elsewhere [29]. *N*-Ethyl-*N′*-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC, Fluka), *N*-hydroxysuccinimide (NHS, Aldrich), phloretic acid (PA, Fluka), glycolic acid (GA, Fluka), hydrogen peroxide (H_2O_2, 30 wt%, Aldrich) and deuterium oxide (D_2O, Aldrich) were purchased with the highest purity available and used without further purification. Horseradish peroxidase (HRP, type VI, 298 purpurogallin unit/mg solid) was purchased from Aldrich and used as-received. Phosphate buffered saline (PBS, pH 7.4) was purchased from B. Braun Co. All other solvents were used as-received. Buffers used were a phosphate buffer (pH 2), acetate buffer (pH 5.0), MES buffer (pH 6.5), PBS buffer (pH 7.4) and a CHES buffer (pH 9.0) all at an ionic strength of 150 mM.

2.2. Synthesis of chitosan conjugates

Chitosan conjugates were prepared in two steps by first reacting chitosan with glycolic acid followed by a reaction with phloretic acid. In both steps the molar ratios of reagents to the NH_2 groups of chitosan were varied to obtain the desired degree of derivatization.

As a typical example, 1.5 g of chitosan (DD = 85% from ^{1}H NMR) was dissolved in 600 mL of water containing GA (1.65 g, 0.022 mol) overnight. To the solution EDAC (6.33 g, 0.033 mol) and NHS (3.99 g, 0.026 mol) were added. The resulting solution was stirred for 48 h at room temperature. The reaction mixture was then neutralized with a 1 M NaOH solution to pH 7. In order to remove unreacted GA, the solution was ultra filtrated (MWCO 30 kDa), first with 50 mM NaCl and then with deionized water. Finally, the glycolic acid grafted chitosan (CH-GA) was freeze-dried. DS: 43 (^{1}H NMR). Yield: 68%. ^{1}H NMR (D_2O/CD_3COOD (95/5 v/v)): δ = 4.9 (chitosan anomeric proton, overlaps with water peak), 3.0 and 3.4–4.2 (chitosan glucopyranose ring protons), 4.2 (NHCOC$\underline{H}_2$OH), 2.0 (NHCOC$\underline{H}_3$).

By changing the molar feed ratio of carboxylic groups of GA to chitosan amino groups from 1:3 to 2.5:1, chitosan derivatives with different DSs of 10, 19 and 43 were obtained. The yield of CH-GA varied from 68 to 95%.

To a solution of 1 g of CH-GA43 dissolved in 100 mL of water, PA (410 mg, 2.5 mmol) was added and stirred overnight. To the resulting solution, EDAC (725 mg, 3.7 mmol) and NHS (345 mg, 3.0 mmol) were added. The mixture was stirred for 48 h at room temperature. The resulting mixture was neutralized with 1 M NaOH and the chitosan derivative CH-GA/PA was isolated using the procedures described above. DS: 10 (^{1}H NMR). Yield: 76%. ^{1}H NMR (D_2O/CD_3COOD (95/5 v/v)): δ = 6.8 and 7.2 ($CH_2CH_2C_6\underline{H}_4OH$), 4.9 (chitosan anomeric proton, overlaps with water peak), 3.0 and 3.4–4.2 (chitosan glucopyranose ring protons), 4.2 (NHCOC$\underline{H}_2$OH), 2.4–2.9 ($C\underline{H}_2C\underline{H}_2C_6H_4OH$), 2.0 (NHCOC$\underline{H}_3$).

2.3. Characterization

^{1}H NMR (300 MHz) spectra were recorded on a Varian Inova spectrometer (Varian, Palo Alto, USA). Samples of 10–20 mg each were dissolved in 0.8 mL of D_2O containing 5% (v/v) CD_3COOD. The signals of solvent residues were used as reference for the ^{1}H NMR chemical shifts and were set at δ 4.79 for water.

The degrees of substitution (DSs) of GA and PA, defined as the number of substituted NH_2 groups per 100 glucopyranose rings of chitosan, were determined using ^{1}H NMR by comparing the integrals of signals at δ 2.0 (acetamide groups of chitosan) with δ 4.2 (methylene protons of GA) for CH-GA, and δ 6.5–7.5 (aromatic protons of PA) for CH-GA/PA, respectively. To indicate the DS of GA and PA, the samples are coded by adding numbers to the CH-GA/PA polymer as presented in Table 1.

2.4. pH dependent solubility

The solubility of unmodified and modified chitosan at a fixed concentration and different pH values was measured according to a procedure described in literature [11]. Briefly, chitosan, CH-GA and CH-GA/PA were separately dissolved in water (1 mg/mL) and the pH was adjusted to 3 with 0.1 M HCl to give clear solutions. A solution of 0.1 M NaOH was gradually added to the stirred polymer solution until precipitation occurred. The pH value was measured using a pH meter (Metrohm 702 SM Titrino). Upon addition of 0.1 M of NaOH the concentration of the chitosan and its derivatives did not decrease below 70% of the original concentration (1 mg/mL).

2.5. Hydrogel formation

Hydrogel samples (0.5 mL) were prepared in vials at 37 °C. In a typical procedure, to 400 μL of a 1.25 wt% solution of CH-GA43/PA10 in PBS, a freshly prepared mixture of H_2O_2 (38.5 μL of a 0.075 wt% stock solution) and HRP (61.5 μL of a 15.6 μg/mL stock solution) was added and the mixture was gently mixed. The final concentration of CH-GA43/PA10 was 1 wt%. The time to form a gel (denoted as gelation time) was determined using the vial tilting method. No flow within 1 min upon inverting the vial was regarded as the gel state. In all experiments using different concentrations of CH-GA43/PA10 always 0.5 mg HRP per mmol phenol groups and a H_2O_2/phenol molar ratio of 0.5 were applied.

2.6. Gel content and water uptake

To determine the gel content, samples of about 0.5 g of a hydrogel made from CH-GA43/PA10 were lyophilized and weighted (W_d). The dry hydrogels were then extensively extracted with 6 mL of a 1% (v/v) acetic acid solution at room temperature for 3 days to remove uncrosslinked polymer. The solution was replaced each day. The samples were subsequently washed 3 times with deionized water and lyophilized (W_g). The gel content was expressed as $W_g/W_d \times 100\%$.

Dried hydrogel samples were immersed in 2 mL buffer solutions having pH values of 2.0, 5.0, 6.5, 7.4 or 9.0 at 37 °C for 1 day in order to reach equilibrium

Table 1
Preparation of chitosan-based conjugates.[a]

Sample code	[GA]/[NH_2] feed ratio	DS[b] (GA or PA)	Yield	Maximum pH for solubilization[c]
CH-GA10	0.3	10	95%	7
CH-GA19	1.0	19	82%	8
CH-GA43	2.5	43	68%	10
CH-GA43/PA10	0.5	10	76%	10

[a] Polymer concentration was 10 mg/mL H_2O, molar ratio of EDAC/NHS/COOH = 1.5/1.2/1.
[b] Degree of substitution determined from ^{1}H NMR.
[c] Polymer concentration was 1 mg/mL.

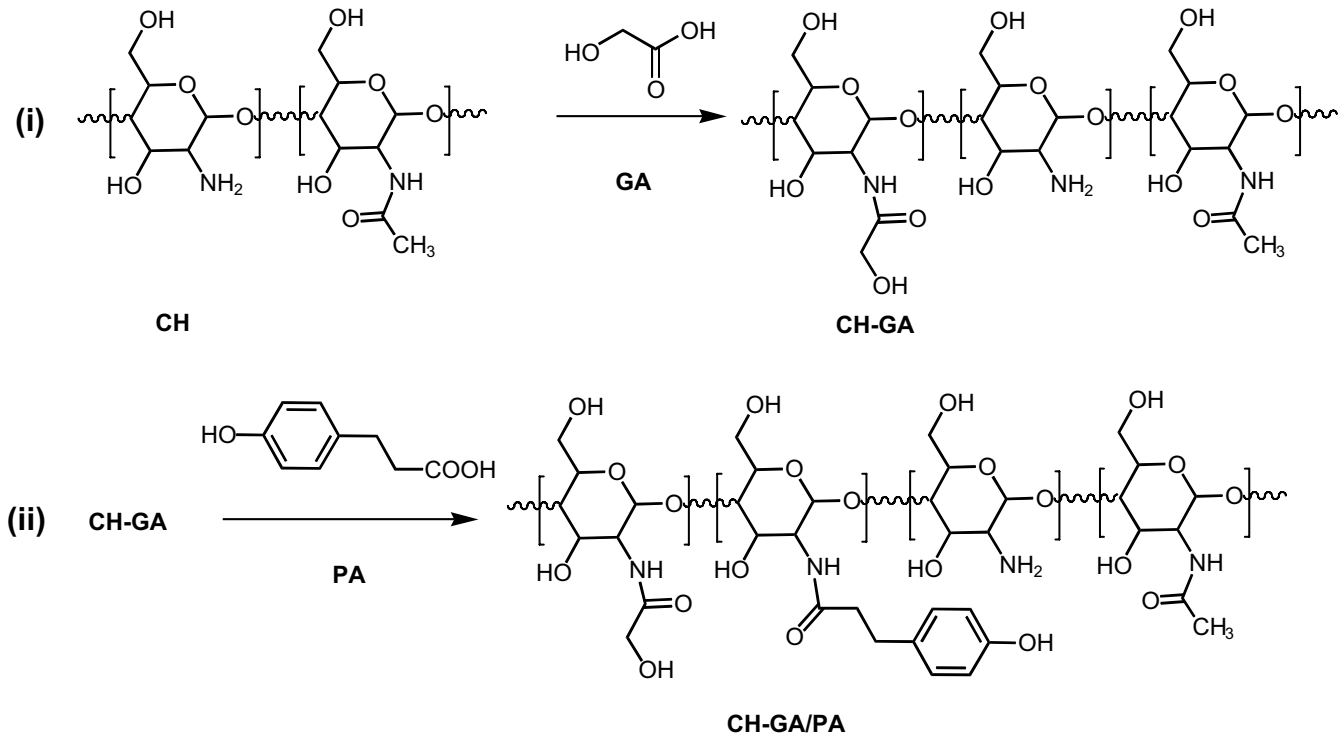

Fig. 1. Synthesis of glycolic acid grafted chitosan (CH-GA) and subsequent conjugation of phloretic acid (PA) to give CH-GA/PA.

swelling. After removal of surface water, the samples were weighted (W_s). The water uptake of dried hydrogels was expressed as $(W_s - W_g)/W_g \times 100\%$ [30].

2.7. In vitro degradation

Hydrogel samples (about 0.5 g) were prepared in vials according to the procedure described in Section 2.5 and accurately weighted (W_i). Subsequently, 2 mL of PBS solutions containing different concentrations of lysozyme (0.5–2 mg/mL) were applied on top of the hydrogels and then incubated at 37 °C. At regular time intervals, the buffer solution was removed from the samples and the hydrogels were weighted (W_t). The percentage of original gel weight remaining is expressed as $W_t/W_i \times 100\%$. The medium was replaced twice a week and the experiments were performed in triplicate.

2.8. Rheological analysis

Rheological experiments were carried out with an MCR 301 rheometer (Anton Paar) using parallel plates (25 mm diameter, 0°) configuration at 37 °C in the oscillatory mode. In a typical example, 77 μL of a H_2O_2 stock solution (0.075 wt%, in PBS) and 123 μL of the HRP stock solution (15.6 μg/mL, in PBS) were mixed. The HRP/H_2O_2 solution was then immediately mixed with 800 μL of a solution of CH-GA43/PA10 (1.25 wt%, in PBS) using a double syringe (2.5 mL, 1:4 volume ratio) equipped with a mixing chamber (Mixpac). After the samples were applied to the rheometer, the upper plate was immediately lowered to a measuring gap size of 0.5 mm, and the measurement was started. To prevent evaporation, a layer of oil was introduced around the polymer sample. The evolution of the storage (G') and loss (G'') moduli was recorded as a function of time. A frequency of 0.5 Hz and a strain of 0.1% were applied in order to maintain a linear viscoelastic regime.

2.9. Chondrocyte isolation and encapsulation

Bovine cartilage was harvested from the patellar–femoral groove of calf legs. Cartilage tissue was cut into small pieces and chondrocytes were isolated by incubation in Dulbecco's modified Eagle's medium (Gibco) (DMEM) containing 0.2% collagenase type II at 37 °C for 8 h. The isolated chondrocytes were washed, centrifuged and resuspended in DMEM with 10% heat inactivated fetal bovine serum, 1% Penicillin/Streptomycin (Gibco), 0.5 mg/mL fungizone (Gibco), 0.01 M MEM nonessential amino acids (Gibco), 10 mM HEPES and 0.04 mM L-proline. The cell suspension was then seeded in culture flasks and incubated at 37 °C in humidified atmosphere (95% air/5% CO_2). At confluence, the cells were detached using 0.25 wt% trypsin in PBS, resuspended in PBS, and used for the experiments.

To encapsulate the chondrocytes, a stock PBS solution of 2.5 wt% CH-GA43/PA10 was first exposed to UV (Bio-Rad Gel Doc 2000) for half an hour. HRP and H_2O_2 stock solutions were made in PBS and sterilized by filtration through filters with a pore size of 0.22 μm. Chondrocytes were incorporated in the hydrogels using the same procedure as for the gel formation in the absence of cells. Briefly, 61.5 μL of the HRP stock solution was added into 38.5 μL of the H_2O_2 stock solution to obtain solutions containing HRP and H_2O_2. Chondrocyte/CH-GA43/PA10 suspensions were prepared by mixing 200 μL of polymer solutions with 200 μL of medium containing chondrocytes. To 100 μL of cell/polymer suspensions, 25 μL HRP/H_2O_2 mixtures were added and the hydrogel precursor was gently mixed. Before gelation, the precursor was quickly transferred onto cover slides in the culture plate. The final concentration of CH-GA43/PA10 was 1 wt% and the cell seeding density in the gels was 5×10^6/mL. The constructs were cultured in the previously described medium, standard for chondrocyte expansion. The gels were incubated at 37 °C and 5% CO_2, and the medium was replaced every 2 or 3 days.

2.10. Cytotoxicity assay

A viability study on hydrogel encapsulated chondrocytes was performed with a Live–dead assay and the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-

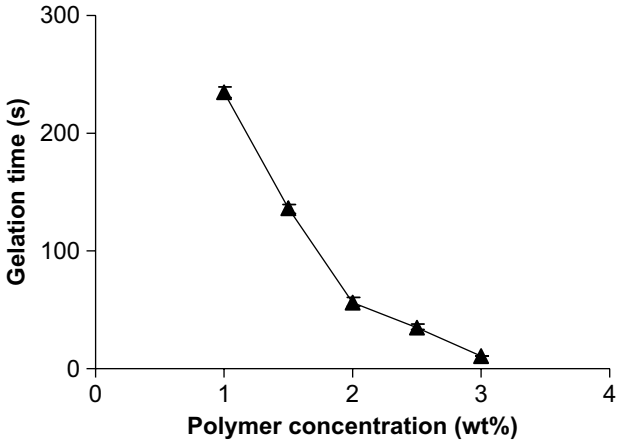

Fig. 2. Gelation times of CH-GA43/PA10 hydrogels as a function of polymer concentration. Reaction conditions: molar ratio of H_2O_2/phenol is 0.5; 0.5 mg HRP per mmol phenol groups; 37 °C, PBS.

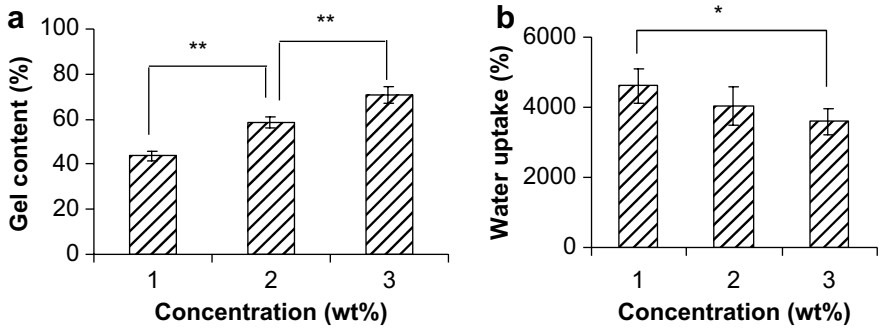

Fig. 3. Gel content (a) and equilibrium water uptake (b) of extracted CH-GA43/PA10 hydrogels as a function of initial polymer concentration at pH 7.4 and 37 °C (Student's *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$).

tetrazolium bromide) assay. At days 1, 3, 7, and 14, the hydrogel constructs were rinsed with PBS and stained with calcein/ethidium homodimer using the Live–dead assay Kit (Invitrogen), according to the manufactures' instructions. Agarose hydrogel/cell constructs (0.5 wt%) with the same chondrocyte density were used as a control. Hydrogel/cell constructs were visualized using fluorescence microscopy (Zeiss). As a result living cells fluoresce green and the nuclei of dead cells red. MTT staining was performed using 1% (total medium volume) of MTT solution (5 mg/mL, Gibco) and an incubation time of 2 h. Hydrogel/cell constructs were then visualized using a light microscope. The morphology of the chondrocytes in the hydrogels was studied using a Philips XL 30 ESEM-FEG scanning electron microscopy (SEM) operating at a voltage of 10 kV. After 14 days' *in vitro* culturing the hydrogel/cell constructs were fixed with formalin by sequential dehydration and critical point drying. These samples were gold sputtered (Carringdon) and analyzed with SEM.

3. Results and discussion

3.1. Synthesis and characterization of chitosan conjugates

Chitosan is an insoluble material in neutral aqueous solutions. To increase its water solubility chitosan was modified by reacting glycolic acid, GA, with the primary amino groups of chitosan using EDAC/NHS activation (Fig. 1). The reaction was performed in diluted GA solutions with different molar feed ratios of GA to amino groups of chitosan and without adding other acidic components since chitosan readily dissolved in the acidic GA solution having a pH value of approximately 4. After 2 days reaction, the resulting chitosan derivatives (CH-GA) were purified by ultrafiltration and isolated after freeze-drying. The ^{1}H NMR spectrum of CH-GA showed that a new signal at δ 4.2 was present corresponding to the methylene protons of the glycolamide units. The degree of substitution (DS) of GA, defined as the number of glycolamide moieties per 100 glucopyranose rings of chitosan, was determined from the ^{1}H NMR spectra by comparing the integrals of signals at δ 2.0 and 4.2, attributed to the protons of acetamide group ($NHCOC\underline{H}_3$) in chitosan and methylene protons ($COC\underline{H}_2OH$) in GA moieties, respectively. The DS of CH-GA increased from 10 to 43 when the [GA]/[NH_2] feed ratio was increased from 0.3 to 2.5 (Table 1). The pH dependent solubility of the CH-GA polymers was evaluated by titration of a 1 mg/mL polymer solution with a NaOH solution. The pH value at which the CH-GA started to precipitate was used as an indication of the water solubility of the chitosan derivatives. Native chitosan with a degree of deacetylation of 85% dissolves in water (1 mg/mL) up to a maximum pH of 6.4. The limited solubility at higher pH values is due to the formation of intra macromolecular and inter chain hydrogen bonds between the amino and hydroxyl groups [31]. By converting the free amine groups to glycolamide groups the intermolecular H-bonds will be disrupted, which improves the water solubility of the chitosan derivatives. It was found that the solubility of the CH-GAs at a concentration of 1 mg/mL increased with an increase in the degree of substitution of GA to the chitosan backbone. The CH-GA19 was soluble up to pH 7 while CH-GA43 was still soluble up to a maximum pH value of 10 (Table 1).

Based on these results, chitosan derivatives CH-GA43 were selected for further modification. Phloretic acid (PA) was then coupled by activation of its carboxylic acid group using EDAC/NHS and subsequent reaction with the remaining amine groups of CH-GA43. The extent of coupling of PA moieties was followed by ^{1}H NMR using the new peaks at δ 6.8 and 7.2 of the aromatic protons of the phenol groups. Accordingly, the degree of substitution of PA was determined by comparing the integral ratios of the phenol and *N*-acetyl proton signals. A degree of substitution of 10 was obtained when the molar feeding ratio of carboxylic acid groups of PA to chitosan amino groups was 0.5. At a concentration of 1 mg/mL the CH-GA43/PA10 was soluble up to a pH value of 10 and could be easily dissolved in water or PBS at a polymer concentration of 30 mg/mL.

3.2. Hydrogel formation and gelation time

Fast in-situ gelation is required in order to maintain the cells and bioactive molecules at the injection site. We previously showed that the enzymatic crosslinking of dextran–tyramine conjugates using HRP and H_2O_2 provided a fast and efficient way to obtain an in-situ forming hydrogel under physiological conditions [28]. Moreover, the gelation time of dextran–tyramine conjugates could be adjusted by changing the ratios of HRP and H_2O_2 to phenol groups, and the polymer concentration. Using a 1 wt% CH-GA43/PA10 solution in PBS, 0.5 mg HRP per mmol phenol groups and a H_2O_2/phenol molar ratio of 0.5, a hydrogel was formed in approximately 4 min as determined by the vial tilting method.

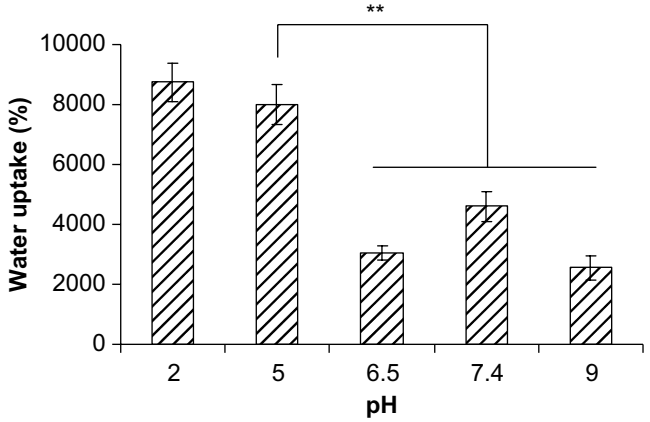

Fig. 4. Water uptake of extracted CH-GA43/PA10 hydrogels after swelling in buffers with different pH values at 37 °C (Student's *t*-test, $^{**}p < 0.01$).

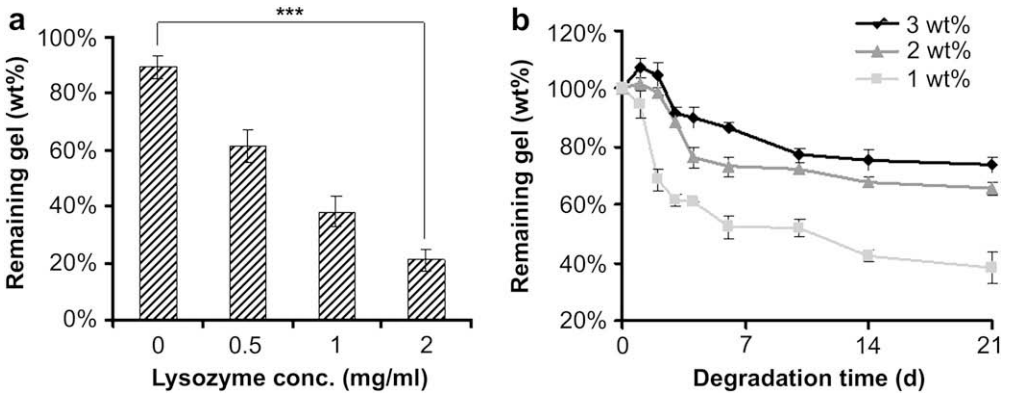

Fig. 5. Enzymatic degradation of (a) 1 wt% CH-GA43/PA10 hydrogels after incubation in PBS with lysozyme concentrations from 0 to 2 mg/mL for 21 days at 37 °C; (b) Degradation of CH-GA43/PA10 hydrogels in PBS containing 1 mg/mL lysozyme as a function time for different initial polymer concentrations at 37 °C (Student's *t*-test, $^{***}p < 0.001$).

Increasing the polymer concentration from 1 to 3 wt%, and maintaining the other reaction conditions the same revealed a large decrease in gelation time from 4 min to 10 s (Fig. 2).

3.3. Gel content and water uptake

The gel content of crosslinked CH-GA43/PA10 hydrogels was determined gravimetrically by extracting the gels with 1% (v/v) acetic acid for 3 days. Fig. 3a shows that the gel content increased with increasing polymer concentrations from 1 wt% to 3 wt% and the highest content exceeding 70% was obtained at a polymer concentration of 3 wt%. This indicates that network formation is more efficient at higher polymer concentrations.

The ability to hold sufficient amounts of water inside the network structure is an important parameter to characterize hydrogels. The effect of the initial polymer concentration on the water uptake of hydrogels is shown in Fig. 3b. The water uptake of the extracted CH-GA43/PA10 hydrogels decreased from 4600 to 3600% with increasing initial polymer concentration from 1 to 3 wt%. These results indicate that the networks formed at a higher polymer concentration have a higher crosslink density.

It is known that chitosan amino groups have a pK_a value of 6.5. As a result, the crosslinked chitosan gels were expected to have a pH dependent swelling behavior. The effect of pH on the water uptake of CH-GA43/PA10 hydrogels is presented in Fig. 4. When placed in buffers with pH values ranging from 6.5 to 9, the hydrogels showed a comparable water uptake value. However, water uptake of the gels increased almost twice when the gels were swollen in acidic buffers with a pH value of 2 or 5. The protonation of amino groups leads to electrostatic repulsion and expansion of the polymer chains and consequently more water can diffuse into the network, leading to an increase in water uptake [32].

3.4. Enzymatic degradation of chitosan hydrogels

It has been reported that chitosans undergo enzymatic hydrolysis of the glycosidic bonds of acetylated residues present in the chitosan backbone by lysozyme [13–16]. To mimic an *in vivo* degradation profile, PBS solutions containing different concentrations of lysozyme were applied on top of the CH-GA43/PA10 hydrogels and incubated at 37 °C. The lysozyme concentration ranged from 0.5 mg/mL to 2 mg/mL corresponding with the lysozyme concentration in the extracellular matrix of human cartilage [16]. The degradation of CH-GA43/PA10 hydrogels in the absence or presence of lysozyme was evaluated by determining the remaining gel weight as a function of time. Fig. 5a shows that after 21 days in PBS the weight ratio (degraded gel weight/initial gel weight × 100%) of a 1 wt% CH-GA43/PA10 hydrogel slightly decreased to 90%, whereas the ratio significantly decreased to about 20–60% of the original gel weight in the presence of lysozyme. Moreover, at the same incubation time the gel weight ratios decrease with increasing concentrations of lysozyme from 0.5 mg/mL to 2 mg/mL.

In order to investigate the influence of the initial polymer concentration on the degradation rate, PBS solutions containing 1 mg/mL lysozyme were added to CH-GA43/PA10 hydrogels prepared at different polymer concentrations, and the remaining gel weight in time was determined (Fig. 5b). It is noticed that there was a slight weight increase in the first 2 days for CH-GA43/PA10 hydrogels prepared at concentrations of 2 and 3 wt%, which was a result of a higher degree of swelling after partial degradation of the hydrogels. Moreover, hydrogels prepared at higher polymer concentrations degraded more slowly. For example, about 80% of gel weight remained for the 3 wt% CH-GA43/PA10 hydrogel after 3 weeks, whereas only 40% of gel weight remained for the 1 wt% CH-GA43/PA10 hydrogel after the same time period. *In vitro* experiments using poly(glycolic acid) scaffold showed that chondrocytes started to produce an extracellular matrix (ECM) after 3 days [33]. Moreover, chondrocytes cultured in agarose gels synthesized a mechanically functional ECM in approximately one month [34].

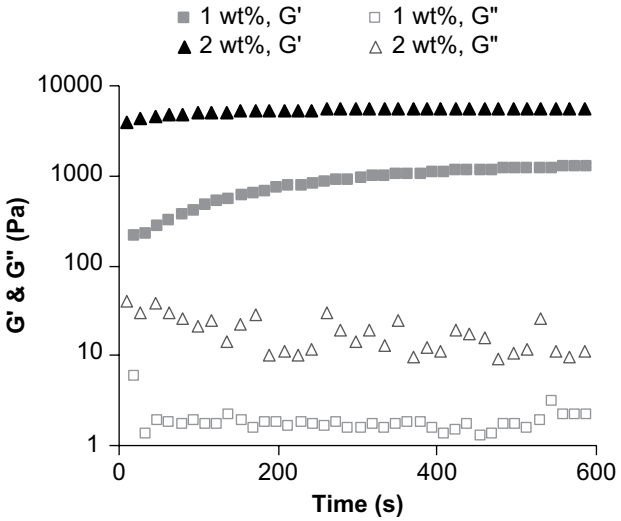

Fig. 6. Storage and loss moduli (G' and G'') of 1 and 2 wt% CH-GA43/PA10 hydrogels as a function of time at 37 °C. A frequency of 0.5 Hz and a strain of 0.1% were applied.

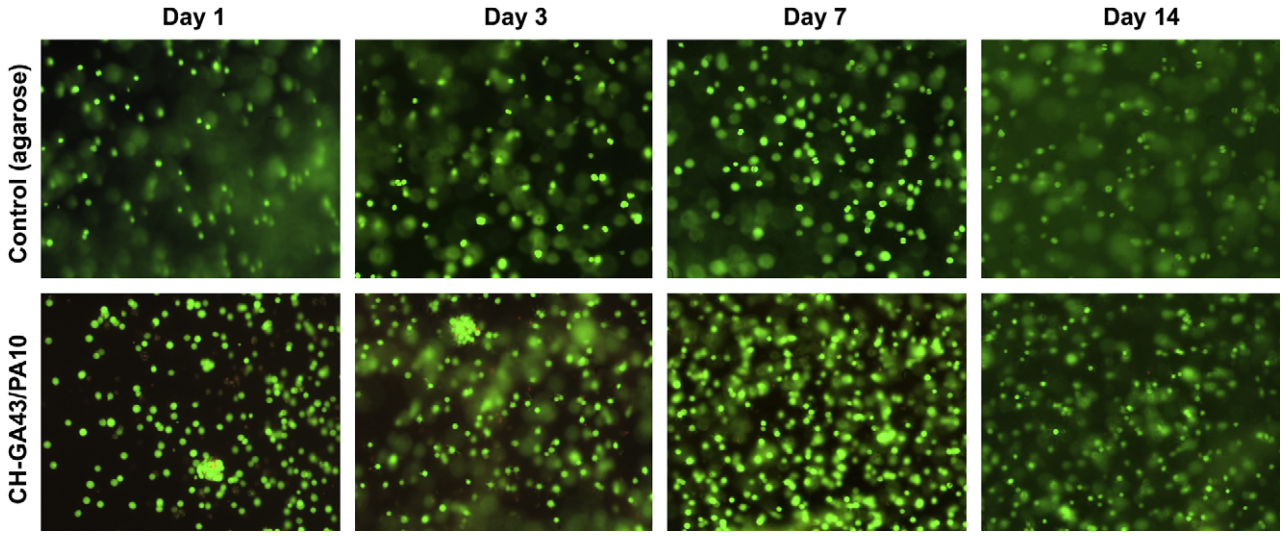

Fig. 7. Live–dead assay showing chondrocytes incorporated in the chitosan hydrogels, after 1, 3, 7 and 14 days in culture. Agarose hydrogels (0.5 wt%) were used as a control. Cells were stained with calcein-AM/ethidium homodimer (dead cells stained red and living cells green) and visualized using fluoresce microscopy. Cell density: 5×10^6/mL.

Based on the preliminary results of the degradation of CH-GA43/PA10 hydrogels, these hydrogels are expected not to be completely degraded within one month.

3.5. Rheological analysis

The mechanical properties of the chitosan-based hydrogels were studied by oscillatory rheology experiments of polymer solutions containing HRP and H_2O_2 in PBS at 37 °C. A solution of CH-GA43/PA10 and a mixture of HRP and H_2O_2 solutions (in PBS) were applied to the rheometer using a double syringe equipped with a mixing chamber.

Generally, after mixing the reactants the storage modulus increased in time due to enzymatic crosslinking reactions of CH-GA43/PA10, until reaching its plateau value, marking the end of the crosslinking process. In Fig. 6 the storage and loss moduli of 1 and 2 wt% chitosan hydrogels are presented. It can be seen that a higher initial polymer concentration resulted in a higher storage modulus of the hydrogels. In addition, the time to reach the plateau value of the storage modulus was also shortened. For example, after 7 min, chitosan hydrogels reached a storage modulus plateau value of 1.3 kPa at 1 wt% polymer concentration, while hydrogels prepared from 2 wt% polymer solutions almost immediately reached a plateau value of 5.5 kPa after starting the measurement. No increases in the storage moduli in time were observed for samples of CH-GA43/PA10 without HRP and H_2O_2 and CH-GA43 with HRP and H_2O_2, indicating that no gelation took place (data not shown). Moreover, the damping factor (defined as G''/G'), calculated from Fig. 6, was between 1×10^{-3} and 2×10^{-3}. The low damping factor indicates that these chitosan hydrogels are highly elastic.

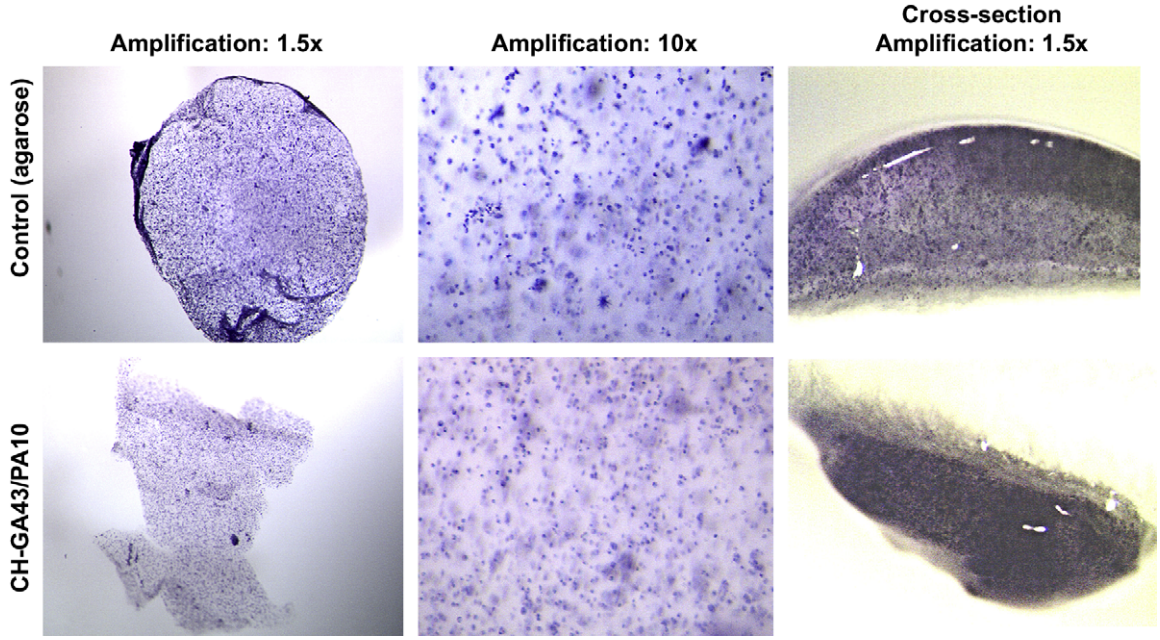

Fig. 8. MTT assay showing the encapsulated chondrocytes in the chitosan hydrogels, after 14 days in culture. Agarose hydrogels (0.5 wt%) were used as a control. Metabolically active cells stain purple and constructs were visualized using light microscopy. Cell density: 5×10^6/mL.

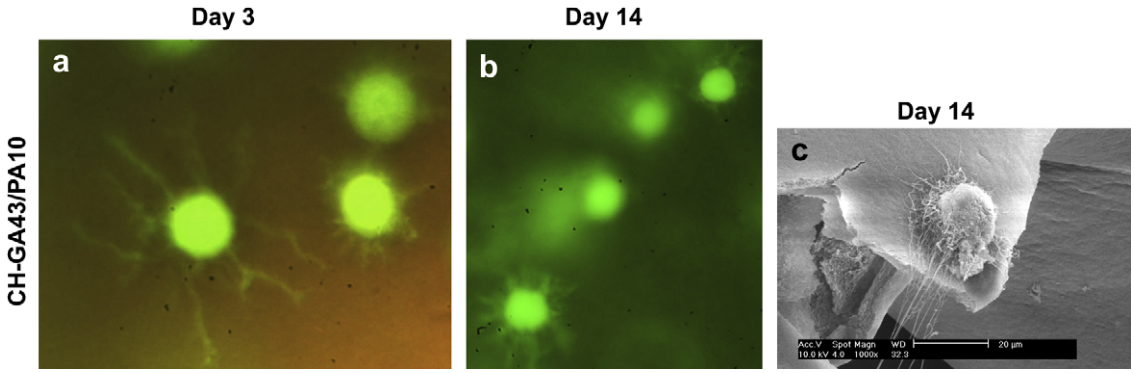

Fig. 9. Fluorescence microscopy images (a and b) and SEM image (c) showing the chondrocyte morphology inside 1 wt% CH-GA43/PA10 hydrogels after 3 and 14 days in culture. For the Live–dead assay, cells were stained with calcein-AM/ethidium homodimer (dead cells stain red and living cells green). Cell density: 5×10^6/mL.

3.6. Chondrocyte incorporation inside chitosan-based hydrogels

In a biocompatibility study of CH-GA43/PA10 hydrogels, chondrocytes were incorporated inside the gels during the gel preparation and the hydrogel/cell constructs were cultured in medium without differentiation factors up to 2 weeks. Cell viability as a function of culture time was determined by using a Live–dead assay kit. The viable (stained green) and dead cells (stained red) were visualized by fluorescence microscopy. Fig. 7 shows that at all culture times, predominantly living cells (>90%) were found within the CH-GA43/PA10 hydrogels. These results show that the hydrogels have a good cytocompatibility. These findings were further confirmed with an MTT assay (Fig. 8). Metabolically active cells which stained purple with the MTT reagent were observed throughout the hydrogel, and a uniform cell distribution was obtained. No apparent differences in the metabolic activity of chondrocytes in chitosan hydrogels and cells in the agarose control gels were detected. These results strongly suggest that the gelation process does not compromise cell viability and that sufficient mass transport of nutrients and oxygen to the cells inside the CH-GA43/PA10 gel matrix takes places. Therefore, the enzymatic crosslinked chitosan hydrogels have a better biocompatibility as compared to other injectable hydrogel systems based on methacrylated chitosan [21].

Cells, scaffolds and signaling factors are the framework of the tissue engineering triad [35]. One of the major challenges for cartilage tissue engineering is enabling the cells to behave within an artificial scaffold as they do in native cartilage. Differentiated chondrocytes are characterized by a round morphology. Preserving this feature inside hydrogels is a prerequisite for efficient chondrogenic matrix production. As observed in Fig. 9a, chondrocytes inside chitosan hydrogels still maintained a round morphology, up to 14 days. Interestingly, inside the analyzed samples the encapsulated chondrocytes appeared to attach on the materials and form clusters after 3 days in culture. The morphology of chondrocytes was further observed by SEM. Fig. 9c shows that chondrocytes retained their round shape after being cultured for 14 days.

4. Conclusions

Water-soluble chitosan derivatives (CH-GA/PA) were conveniently synthesized by a two-step synthesis procedure by the sequential conjugation of glycolic acid (GA) and phloretic acid (PA) to native chitosan using EDAC/NHS. Gelation of the CH-GA43/PA10 was performed using HRP and H_2O_2. Gelation times can be varied from 4 min to 10 s by increasing the polymer concentration from 1 to 3 wt%. The gel content, water uptake, enzymatic degradation rate and mechanical properties could be adjusted by varying the initial polymer concentration. Storage moduli of the gels in the range of 1–5.5 kPa could be obtained. Primary chondrocyte culturing experiments showed that CH-GA43/PA10 hydrogels have a good biocompatibility. These studies have demonstrated that the water-soluble chitosan derivative CH-GA43/PA10 has a high potential as an injectable biomaterial for cartilage tissue engineering.

Acknowledgement

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Appendix

Figures with essential colour discrimination. Certain figures in this article, in particular Figs. 7–9, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2009.01.020.

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