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Covalently Crosslinked Hyaluronic Acid-Chitosan Hydrogel Containing Dexamethasone as an Injectable Scaffold for Soft Tissue Engineering

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ABSTRACT: Injectable hybrid hydrogels were produced by mixing crosslinked aldehyde hyaluronic acid with dexamethasone grafted water soluble chitosan, without the addition of a chemical crosslinking agent. The gelation is attributed to the Schiff-base reaction between amino and aldehyde groups of hydrogel precursors. In this article, the water soluble chitosan, *N*-succinyl-chitosan, grafted with dexamethasone via water soluble carbodiimide chemistry has been characterized. *In vitro* gelation time, morphologies, swelling, weight loss, and compressive modulus of hybrid hydrogels in phosphate buffered saline were studied. The dexamethasone grafted hydrogel showed a slightly lower gelation time, higher water uptake and faster weight loss compared to the hydrogel without dexamethasone. Human adipose-derived stem cells were encapsulated into the dexamethasone grafted hydrogel *in vitro* to assess the biological performance and applicability of the hydrogel as cell carrier. Results demonstrated that the dexamethasone grafted hydrogel resulted in enhanced cell adhesion and proliferation as compared to the hydrogel without dexamethasone. These characteristics provide a potential opportunity for the dexamethasone grafted hybrid hydrogel as an injectable scaffold in adipose tissue engineering applications. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 129: 682–688, 2013

KEYWORDS: biomaterials; biodegradable; gels

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INTRODUCTION

Biodegradable polymers have been used as cell scaffolds, adhesive medical applications and delivery vehicles with promising results.[1–6] Many natural biopolymers, such as collagen, gelatin, fibrinogen, chitosan, and hyaluronic acid (HA), have been used as hydrogel scaffolds for a variety of tissue engineering applications.[7–10] While the versatility of natural polymer hydrogels via chemical crosslinking has been broadly exploited, a major limitation is the intrinsic toxicity of the synthetic schemes and the inability to translate these approaches into biological applications.[11–15]

Recent studies have identified that the Schiff-base reaction can be used to crosslink functional amine and aldehyde groups and present in natural biomaterials with noncytotoxic effects compared to studies performed with commonly used chemical crosslinkers.[16–18] Our laboratory has examined the utilization of the Schiff-base reaction to crosslink natural polymers to form biodegradable hydrogels which has the potential to produce novel scaffolds for soft tissue engineering applications.[19,20] However, for practical soft tissue applications, the challenge is to deliver necessary adipogenic factors such as dexamethasone and insulin to induce adipose-derived stem cells (ASCs) for adipogenesis.[21–23] Although many hydrogels have been served as delivery systems, conventional delivery methods are limited by the intrinsic inability to translate dexamethasone into soft tissue engineering due to the burst release. Dexamethasone that is covalently immobilized into a hydrogel scaffold can be more efficiently transported to a localized site and be released in a sustained-dosage form. To the best of our knowledge, little has been reported on the dexamethasone grafted hydrogel as ASCs carrier for soft tissue engineering. Hererin, we describe a natural HA and chitosan hybrid hydrogel containing covalently grafted dexamethasone for ASCs encapsulation.

Dexamethasone has most commonly been used to treat inflammation and auto-immune diseases as well as is an important factor in adipogenesis.[24] Dexamethasone is one of key adipogenic factors to induce adipogenesis for practical adipose tissue engineering application.[24,25] HA is a naturally nonsulfated glycosaminoglycan that is widely distributed throughout the extracellular matrix (ECM) of all connective tissues in human and other animals. Due to its good biocompatibility and

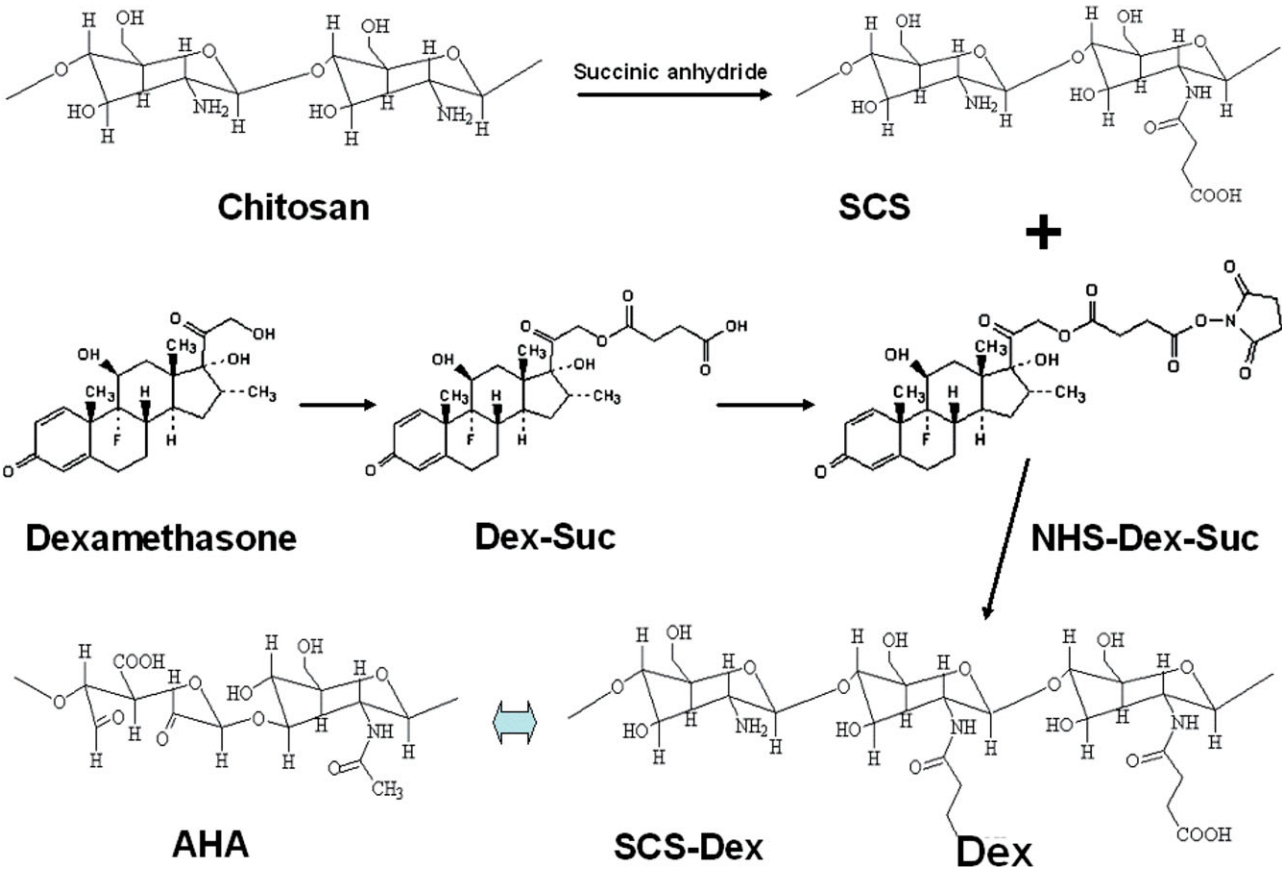

Figure 1. Synthetic route of dexamethasone grafted chitosan derivative, SCS-Dex, which was obtained by introduction of succinyl groups and dexamethasone into the *N*-terminal of the glucosamine units. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

biodegradability, HA shows promise in biomedically-relevant hydrogel systems.[26,27] Chitosan is a partially deacetylated derivative from chitin composed of glucosamine and *N*-acetylglucosamine, which has poor solubility in physiological solvents due to its strong intermolecular hydrogen bonding.[26,27] *N*-succinyl-chitosan (SCS), a water soluble chitosan derivative, was synthesized via introduction of succinyl groups at the *N*-position of the glucosamine units of chitosan, which is attractive material for hydrogel scaffold due to its solubility, biodegradability, and biocompatibility.[19,28,29]

In this study, dexamethasone covalently grafted HA-SCS hydrogels were prepared via the Schiff-base reaction. We investigated that the SCS grafted with dexamethasone to prepare bioactive hydrogel (Figure 1). Gelation time, morphology, swelling ratio, weight loss, and compressive modulus of hydrogel were examined. The hydrogel was then used to culture human ASCs *in vitro* to assess its biological performance and applicability for soft tissue engineering.

EXPERIMENTAL

Materials

HA sodium, chitosan (deacetylation degree: 85%), dexamethasone, 4-dimethylaminopyridine, *N*-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), succinic anhydride, sodium periodate, and *t*-butyl carbazate were purchased from Sigma–Aldrich. CyQuant Cell Proliferation Assay Kit were purchased from Invitrogen, Eugene, OR. All chemicals and reagents were used as received.

Synthesis of Aldehyde Hyaluronic Acid (AHA)

AHA was synthesized according to an already reported procedure.[20] HA (1.0 g) (~2.5 mmol) was dissolved in 100 mL nanopure H_2O at a concentration of 10 mg/mL. An aqueous solution of sodium periodate (0.5*M*, 5 mL) was added dropwise, and the reaction mixture was stirred for 2 h at room temperature in the dark. Ethylene glycol (1 mL) was then added to inactivate any unreacted periodate. The reaction was stirred for 1 h at ambient temperature and the solution was purified by exhaustive dialysis against H_2O for three days, and the dry product was obtained by freeze-drying. The percentage oxidation of AHA was quantified as 45% by measuring the number of aldehydes in the polymer using *t*-butyl carbazate.[29]

Synthesis of *N*-succinyl-chitosan (SCS)

SCS was synthesized according to an already reported procedure.[19] Chitosan (0.5 g) was dissolved in 40 mL 5% (v/v) lactic acid solution and then 160 mL methanol was added to dilute the solution. Succinic anhydride (1.5 g) was added to this solution with stirring at room temperature. After 24 h, the succinyl modified chitosan was precipitated by adjusting the solution pH to 6~7. The precipitate was filtered, redissolved in H_2O, and dialyzed for 3 days. The purified product was freeze-dried and

stored at 4°C. The substitution degree of SCS was determined as 36% by the ninhydrin assay.

Synthesis of Dexamethasone Grafted SCS (SCS-Dex)

Synthesis of dexamethasone-succinate (Dex-Suc) was based on previous reports.[16] Dexamethasone (2.455 g) (6.25 mmoL), 10.52 g of succinic anhydride, and 0.795 g of 4-dimethylaminopyridine were dissolved in 400 mL anhydrous acetone under nitrogen gas. The solution was stirred at room temperature overnight. After the evaporation of acetone, the white crystal was dissolved in 36 mL ethanol, then 85 mL pure water was gradually added. The solution was kept at 4°C for 2 days, and white needle-shaped crystal precipitated. It was filtered and dried under reduced pressure. This reprecipitation was performed twice (yield: 94%). To synthesize *N*-hydroxysuccinimide Dex-Suc (NHS-Dex-Suc), 1.395 g of Dex-Suc, 0.336 g of NHS, and 0.605 g of DCC were dissolved in 80 mL acetone, and stirred for 16 h at room temperature, producing a white crystalline precipitate. The crystals were recovered by filtration, acetone was removed by evaporation, and dry white crystals were obtained and used without further purification (yield: 93%). SCS (100 mg) was dissolved in 15 mL phosphate buffered saline (PBS). NHS-Dex-Suc (130 mg) was dissolved in 30 mL DMF (dimethylformamide). The NHS-Dex-Suc solution was poured into the SCS solution over 30 min and stirred at room temperature for 18 h. The polymer was reprecipitated in 600 mL acetone, then dialyzed against H_2O for 3 days. The purified product was lyophilized then stored at 4°C (yield: 81%). Fourier transformed infrared (FTIR) spectra of SCS and SCS-Dex were measured to confirm the expected pendant functionalities. Samples were recorded with FTIR spectrometer (Nicolet Avatar 360) against a blank KBr pellet background. The substitution degree of dexamethasone was determined as 11% via estimating the remaining amino groups by the ninhydrin assay.[19]

Preparation of AHA-SCS-Dex Hydrogel

AHA and SCS-Dex (2 w/v%) solutions were prepared by dissolving polymer powder in PBS (pH 7.4). The SCS-Dex solution was then crosslinked with AHA solutions at a volumes ratio of 1: 1. The mixture solutions reacted in glass vials and the gelation rate was monitored under sealed reaction conditions. The gelation time was recorded when the solution lost its fluidity at room temperature. The AHA solution was also mixed with SCS solution (2 w/v%, in PBS) to prepare AHA-SCS hydrogels as control samples (volumes ratio 1: 1).

Characterization of Hydrogels

The morphologies of AHA-SCS and AHA-SCS-Dex hybrid hydrogels were characterized by utilizing scanning electron microscopy (SEM) after gelation and subsequent freeze-drying. The hydrogels were lyophilized at −50°C for 24 h and then gold-coated using a Cressington 108 Auto (Cressington, Watford, UK). The cross-sectional morphologies of hydrogels were viewed using a JSM-6330F SEM (JEOL, Peabody) operated at 10 kV accelerating.

To study equilibrium swelling, the freeze-dried hydrogels were weighted and immersed in PBS, and kept at 37°C for 2 h until equilibrium of swelling had been reached. The swollen hydrogels were removed and immediately weighed with a microbalance after the excess of water lying on the surfaces was absorbed with a filter paper. The equilibrium swelling ratio (ESR) was calculated using the following equation: $\mathrm{ESR} = (Ws - Wd)/Wd$, where Ws and Wd are the weights of the hydrogels at the equilibrium swelling state and at the dry state, respectively.

Weight loss of initially weighed hydrogels (W_0) was monitored as a function of incubation time in PBS at 37°C. At specified time intervals, hydrogels were removed from the PBS and weighed (W_t). The weight loss ratio was defined as $100\% \times (W_0 - W_t)/W_0$. The weight remaining ratio was defined as $1 - 100\% \times (W_0 - W_t)/W_0$.

To study compressive modulus, mixtures of solutions described above were injected into a culture plate for 15 min to obtain columned hybrid hydrogels. Compressive modulus of elasticity was measured in the elastic region of hydrogels using a dynamic mechanical analyzer (DMA-7, Perkin–Elmer) in unconfined compression at a constant stress rate of 40 mN min^{-1} up to 20% strain at room temperature.

Cell Isolation

Human ASCs were isolated from human adipose tissue obtained from elective cosmetic surgery procedures performed under the institutional guideline.[15,26] The fat tissues were minced with scissors in the collagenase solution consisted of Hanks' balanced salt solution (3.0 mL g^{-1} of fat) (Sigma–Aldrich, St. Louis, MO), bovine serum albumin (fatty acid free, pH 7.0, 3.5 g/100 mL Hanks') (Intergen Company, Purchase, NY), and 1% type II collagenase (3.0 mg g^{-1} of fat) (Worthington Biochemical Corporation, Lakewood, NJ). The centrifuge tubes were shaken at 100 rpm for 50 min at 37°C. Following digestion, the content of each tube was filtered through a double-layered sterile gauze. The filtrates were then centrifuged at 1000 rpm for 10 min at 37°C, and a 3-layer suspension, consisting of a fatty layer on the top, a serum layer in the middle, and a cellular pellet at the bottom of each tube, was obtained. The fatty layer and most of the supernatant was aspirated off, leaving the pellet intact at the bottom. The pellet in each tube was then suspended in 10 mL of erythrocyte lysis buffer (pH 7.4), vortexed, and centrifuged again at 1000 rpm for 10 min at 37°C. The pellets were suspended in the plating medium consisted of Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 Ham (DMEM/F-12) with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% Fungizone (all products obtained from Gibco, Invitrogen Corporation, Carlsbad, CA). Adherent ASCs were expanded for a period of 5–8 days at 37°C, and the medium was changed every other day until the cells achieved 80% confluence.

Cell Encapsulation

ASC adhesion to the hydrogels was assessed. SCS-Dex and AHA were sterilized under UV irradiation for 1.5 h and then dissolved in sterilized PBS to obtain 2% (w/v) solutions, respectively. SCS-Dex and AHA solutions were injected into the 48-well culture plate and mixed, which were then incubated at 37°C for 15 min to form hybrid hydrogels. DMEM/F12 (1 mL) with 10% FBS and 1% penicillin/streptomycin containing 30,000 cells was added into sample and control wells [polystyrene tissue culture treated wells, tissue culture plate (TCP)]. After 6 h, the number of ASCs attached to the hybrid hydrogels

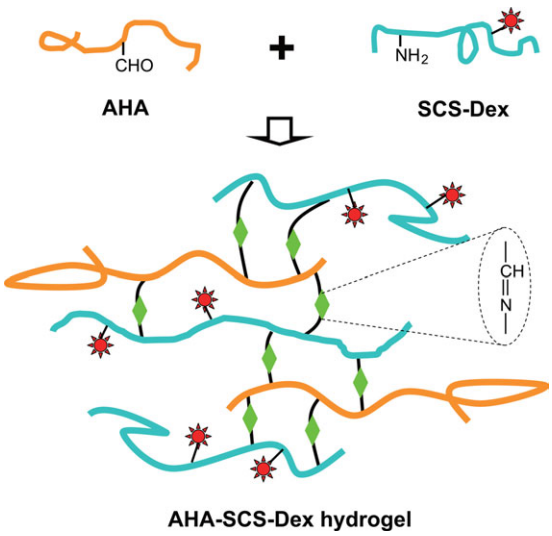

Figure 2. Reaction scheme of hydrogel containing dexamethasone basing the Schiff-base reaction. The hybrid hydrogel was produced by mixing AHA with SCS-Dex. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

was quantified using a CyQuant Cell Proliferation assay. Encapsulation of ASCs within the hybrid hydrogel was also evaluated. SCS-Dex solution was added into a centrifugal tube containing ASCs. After sufficient mixing, the cell containing SCS-Dex solutions were injected into 24-well culture plate to crosslink with AHA solutions. The mixture with cells was then incubated at 37°C to form a cell/gel matrix. The cell density was 5×10^6 mL^{-1} hydrogel. After 15 min, DMEM/F12/10%FBS solution was added into each well. The cell was also encapsulated within AHA-SCS hydrogels as control samples. The number of ASCs in the hybrid hydrogels was quantified using the CyQuant Cell Proliferation assay.

Statistical Analysis

The experimental data from all the studies were analyzed using analysis of variance. Statistical significance was set to P-value ≤ 0.05. Results are presented as mean $\pm$ standard deviation.

RESULTS AND DISCUSSION

Hydrogel Formation

An injectable hydrogel is clinically desired as this system can be used for cell delivery and result in minimally invasive surgeries. Hydrogels derived from naturally occurring polysaccharides mimic many features of ECM and thus have the potential to direct the growth of encapsulated and transplanted cells during tissue regeneration.[29] In this study, an injectable hybrid hydrogel containing dexamethasone was crosslinked AHA with SCS-Dex upon mixing, without the addition of a chemical crosslinking agent. The gelation was attributed to the Schiff-base reaction between amino and aldehyde groups of AHA and SCS-Dex (Figure 2).

Chitosan derivatives were characterized by FTIR (Figure 3). By comparing with chitosan [Figure 3(a)], the spectrum of SCS showed a new absorption peak around 1733 cm^{-1} [Figure 3(b)], which corresponded to the carboxylic group.[19] In the spectrum of the SCS-Dex, the characteristic absorption bonds at 3380, 1806, and 1761 cm^{-1} emerged in the spectrum [Figure 3(c)], which should be assigned to the stretching vibration of O—H and —C=O bands of dexamethasone, respectively. This result demonstrated that dexamethasone has been successfully grafted.

The gelation rate of AHA-SCS-Dex hybrid hydrogel showed a slightly lower gelation rate than the AHA-SCS hydrogel ($P > 0.05$). The gelation time of AHA-SCS and AHA-SCS-Dex hydrogels were 1.8 and 2.1 min, respectively. The grafting of dexamethasone may in turn affect the ability of amine groups to react with aldehyde groups. The cross-sectional images of the AHA-SCS and AHA-SCS-Dex hydrogels were shown in Figure 4. During lyophilized hydrogel fabrication, porous structures were obtained in both of the AHA-SCS and AHA-SCS-Dex hydrogels. Pores evenly distributed throughout the AHA-SCS and AHA-SCS-Dex hydrogels, which showed inter-linked and round structures. Compared to the AHA-SCS hydrogel, the AHA-SCS-Dex hydrogel showed larger pore diameters and loose structure due to the comparatively lower crosslinking density. The formulation of AHA-SCS-Dex hydrogel has less amino groups per unit mass and requires additional time to form Schiff-base crosslink bonds between the SCS-Dex and AHA molecules, which resulted in a longer gelation time and loose structure. Nevertheless, the cross-sectional images of the AHA-SCS and AHA-SCS-Dex hydrogels revealed inter-linked porous scaffolds, which could be beneficial to the swelling and water uptake.

Hydrogel Properties

Swelling property of the hydrogels is crucial for substance exchange when they are used as injectable scaffolds for tissue engineering applications.[29] Both HA and chitosan have an abundant number of hydrophilic groups, such as amino, hydroxyl, and carboxyl groups, which can easily produce hydration with water. The swelling of AHA-SCS and AHA-SCS-Dex hydrogels was determined by examination of mass increases after immersion in PBS at various time points. The result demonstrated that the grafting of dexamethasone significantly influenced the swelling of hydrogels. As shown in Figure 5, the initial swelling ratio of AHA-SCS-Dex hydrogels was significantly higher than the AHA-SCS hydrogels ($P < 0.05$). The AHA-SCS and AHA-SCS-Dex dydrogels showed a slight increase in water uptake before 5 and 7 days, respectively; then values

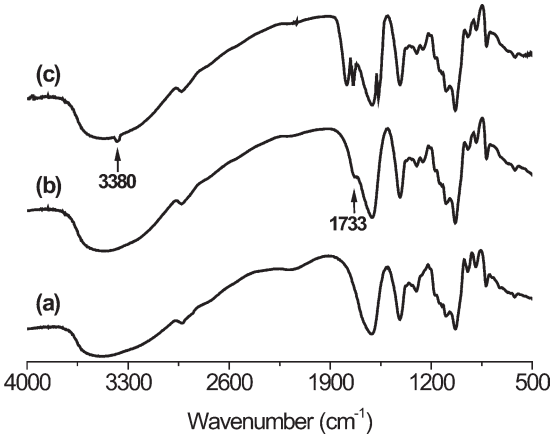

Figure 3. FTIR spectra of (a) chitosan, (b) SCS, and (c) SCS-Dex.

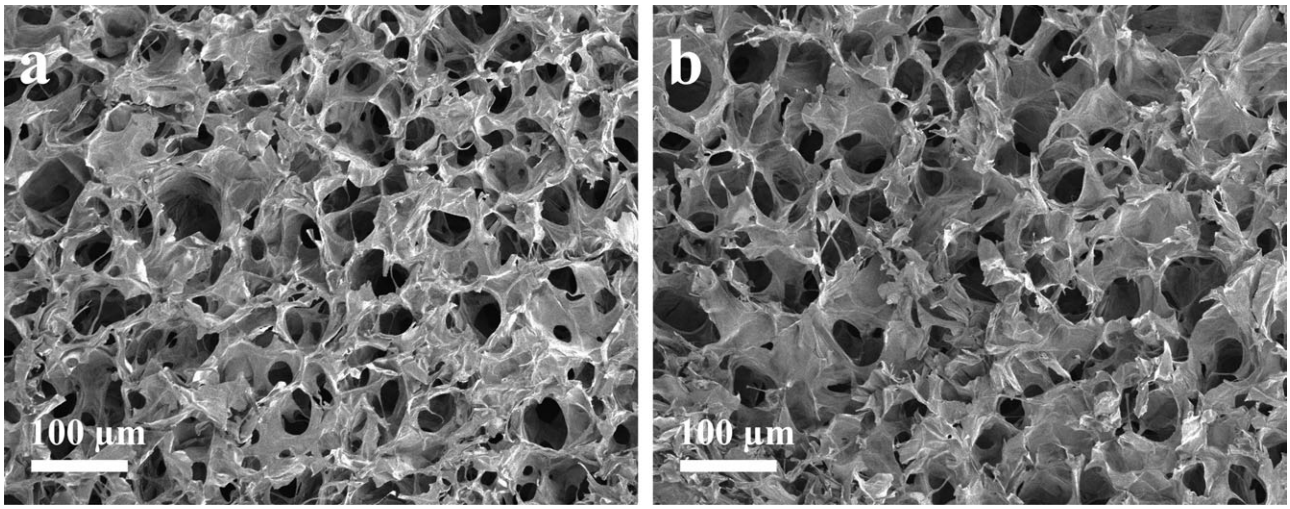

Figure 4. Cross-sectional morphologies of (a) AHA-SCS and (b) AHA-SCS-Dex hydrogels. Volume ratio of precursors was 1: 1.

changed steadily up to 14 days checked so far. For all incubation times, the swelling ratio of AHA-SCS-Dex hydrogels was significantly higher than AHA-SCS hydrogels ($P < 0.05$).

Hydrogels used for adipose tissue engineering should be biodegradable. The degradation behavior of an injectable hydrogel scaffold has a crucial impact on the long-term performance of a cell/gel construct. Therefore, it is important to understand the degradation profile of the hybrid hydrogel. Weight loss of AHA-SCS and AHA-SCS-Dex hydrogels was monitored as a function of time in PBS at 37°C, as shown in Figure 6. The weight loss of AHA-SCS and AHA-SCS-Dex hydrogels increased along with incubation time, with a faster rate of degradation at the initial stage, followed by a slower degradation. The AHA-SCS-Dex hydrogel showed a slightly rapid weight loss comparing to the AHA-SCS hydrogel, which was also likely due to the change of scaffold structure after dexamethasone grafting. The AHA-SCS-Dex hydrogel with higher swelling ratio was more degradable because more involved water would attack the network chains during degradation. At day 14, the weight remaining ratios of AHA-SCS and AHA-SCS-Dex hydrogels were 91 and 87%, respectively.

Compressive modulus of hydrogel scaffold is particularly important for adipose tissue engineering.[20] Compressive modulus of the AHA-SCS and AHA-SCS-Dex hydrogels was studied by a dynamic mechanical analysis method. The AHA-SCS-Dex hydrogel showed a higher compressive modulus than the AHA-SCS hydrogel ($P > 0.05$), which were 23.7 and 18.5 kPa, respectively. Since the compressive modulus of AHA-SCS-Dex hydrogel was very close to that of ECM of natural adipose, however, measures must be taken in the future to further improve the mechanical strength of the present systems if it is used as the injectable scaffold for soft tissue restoration. Therefore, the grafting of dexamethasone resulted in a difference in hydrogel microstructures, which influenced many of macroscopic properties of hydrogels, such as water content, weight loss, and compressive modulus.

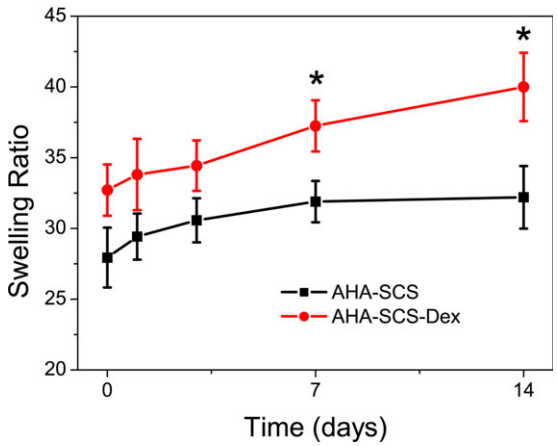

Figure 5. ESR of hybrid hydrogels as a function of incubation time in PBS at 37°C for 14 days. Values reported are an average $n = 5$, ±standard deviation. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

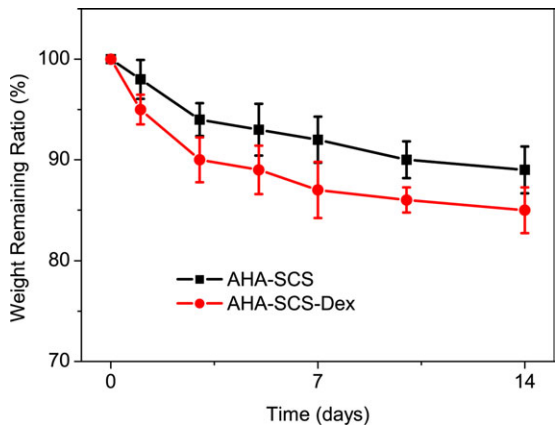

Figure 6. Weight loss of hybrid hydrogels as a function of incubation time in PBS at 37°C. Values reported are an average $n = 5$, ±standard deviation. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

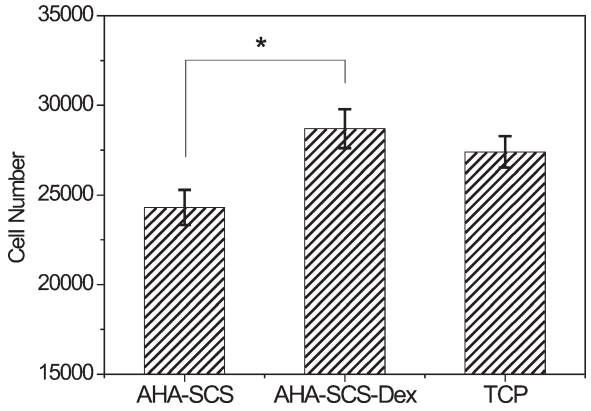

Figure 7. Number of ASCs adhered to surface of hybrid hydrogels versus control wells. Values reported are an average $n = 5$, ±standard deviation. Cell seeding density: 30,000/well (48-well cell culture plate).

Cytoviability of Hydrogels

Another important issue for hydrogel scaffold is the encapsulation efficiency for target cells. The adhesion of human ASCs to AHA-SCS and AHA-SCS-Dex hydrogels was characterized. As shown in Figure 7, after 6 h of incubation, the cell number on the AHA-SCS-Dex surface was significantly greater than that of AHA-SCS hydrogel ($P < 0.05$). This study also indicated that there was no significant difference in the number of cells adhering to the surface of the AHA-SCS-Dex hydrogel compared to the positive control, TCP ($P > 0.05$). Therefore, the AHA-SCS-Dex hydrogel was more favorable for ASC attachment due to its higher bioactivities than the AHA-SCS hydrogel.

Figure 8 showed the time course of changes in relative DNA content of the cells in AHA-SCS and AHA-SCS-Dex hydrogels. The DNA content in the AHA-SCS-Dex hydrogel progressively inreased comparing with initial DNA content during incubation. Furthermore, the DNA content of the AHA-SCS-Dex hydrogel was significantly increased after 5 days of culture ($P < 0.05$). As for the AHA-SCS hydrogel, there was no significant change of DNA number in the 5 days of culture ($P > 0.05$). This study indicated that the AHA-SCS-Dex hydrogels supported ASC survival and proliferation, which may has potential uses in soft tissue engineering applications. However, further optimization of this system is required to promote cell differentiation and ECM production in addition to maintenance of their phenotype.

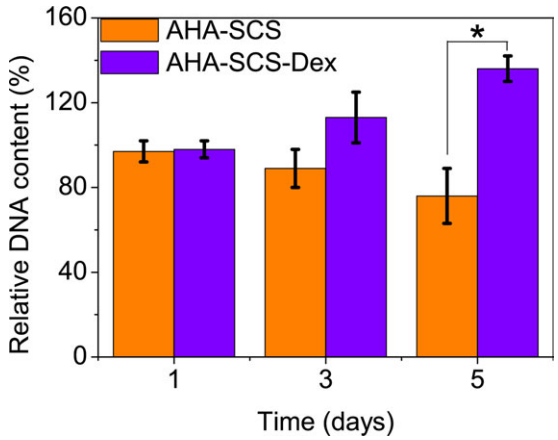

Figure 8. Proliferation of ASCs encapsulated in hybrid hydrogels as a function of culture time. Values reported are an average $n = 5$, ±standard deviation. Cell seeding density: 5×10^6 mL^{-1}. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

CONCLUSIONS

The dexamethasone grafted AHA-SCS hybrid hydrogel was synthesized by using the Schiff-base reaction. The AHA-SCS-Dex hydrogel showed a slightly longer gelation time, higher water uptake and faster weight loss than those of the AHA-SCS hydrogel *in vitro*. The results revealed that the grafting of dexamethasone was an important factor in hydrogel bioactivities. Human ASCs culture study indicated that the AHA-SCS-Dex hydrogel was able to support cell adhesion and proliferation. As this process of hydrogel formation was usually performed under mild conditions without using any extraneous toxic crosslinking agents, we believe that such a composite matrix will has potential opportunity to be used as an injectable cell carrier for clinical adipose, bone, cartilage, and muscle regeneration.

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