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Thermo-sensitive alginate-based injectable hydrogel for tissue engineering

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ABSTRACT

A thermo-sensitive comb-like copolymer was synthesized by grafting PNIPAAm-COOH with a single carboxy end group onto aminated alginate (AAlg) through amide bond linkages. In the copolymer, alginate was the backbone and poly(N-isopropylacrylamide) (PNIPAAm) was the pendant group. The structures of AAlg and three AAlg-g-PNIPAAm copolymers with different PNIPAAm grafting ratios were determined by FTIR and ^{1}H NMR. The rheological properties of AAlg-g-PNIPAAm copolymer hydrogels were measured by monitoring the viscosity, storage modulus and loss modulus as a function of temperature. The lower critical solution temperature of AAlg-g-PNIPAAm copolymers was measured as 35 °C through rheological analysis. An *in vitro* degradation study was carried out by monitoring weight loss. It was confirmed that degradation can be controlled by PNIPAAm modification. Encapsulation of human bone mesenchymal stem cells (hBMSCs) within hydrogels showed that the AAlg-g-PNIPAAm copolymer was not cytotoxic and preserved the viability of the entrapped cells well. The thermo-sensitive AAlg-g-PNIPAAm copolymer has attractive properties that make it suitable as cell or pharmaceutical delivery vehicles for a variety of tissue engineering applications.

1. Introduction

In recent years, injectable hydrogels have attracted more and more attention in the field of biomedical applications because of their easy administration, the minimally invasive procedures associated with their site specific introduction and patient convenience. In particular, injectable hydrogels are extensively studied because of their ability to form three-dimensional (3D) networks under mild conditions. They are attractive carriers for drug delivery, especially for proteins and cells (Cho et al., 2004; Hou, De Bank, & Shakesheff, 2004; Klouda & Mikos, 2008; H.P. Tan et al., 2009; Tan, R. W., Niu, Gan, & Feng, 2009; Wu et al., 2009). Usually, injectable hydrogels are formed by physical reactions (Chenite et al., 2000; Cho et al., 2004) (e.g., phase transition), chemical crosslinkers (Nam, Kimura, & Kishida, 2007; R.W. Tan et al., 2009), photo-cross-linking reactions (Fisher, Dean, & Mikos, 2002; Seiffert, Oppermann, & Saalwaechter, 2007), or enzymatic cross-linking reactions (Chen, Embree, Brown, Taylor, & Payne, 2003; Crescenzi, Francescangeli, & Taglienti, 2002). Most notably, injectable hydrogels formed by temperature-responsive phase transition have attracted more and more attention because gelation can be realized simply as the temperature increases above the lower critical solution temperature (LCST), which is designed below body temperature (Jeong, Kim, & Bae, 2002; H.P. Tan et al., 2009).

Alginate is a well-known natural polysaccharide with negative charge, it is composed of 1,4-linked β-D-mannuronate (MM-blocks) and 1,4-linked α-L-guluronate (GG-blocks) residues in variable proportions. Alginate hydrogel can be formed in the presence of divalent cations, such as Ca^{2+} (Boontheekul, Kong, & Mooney, 2005). Alginate hydrogels have been used as scaffolds for tissue engineering, as delivery vehicles for drugs, and as model extracellular matrices for basic biological studies (H.P. Tan et al., 2009; R.W. Tan et al., 2009; Tonnesen & Karlsen, 2002). However, the degradation of typical alginate hydrogels is very slow and poorly controlled (Boontheekul et al., 2005; Tan et al., 2010), In addition, the cells cannot readily interact with alginates due to the absence of cell-surface receptors which allow binding to alginates as well as the lack of protein adsorption onto alginates (Boontheekul et al., 2005), so the alginate chains were chemically modified to present cell adhesion ligands (Alsberg, Anderson, Albeiruti, Rowley, & Mooney, 2002; Kreeger, Woodruff, & Shea, 2003; Yang, Xie, & He, 2011) or improved to allow better controlled degradation (Li et al., 2010; Yang et al., 2011).

Poly(N-isopropylacrylamide) (PNIPAAm), a typical thermo-sensitive polymer, remains in a soluble state in aqueous solution below its LCST, but forms a hydrogel above this temperature (Jeong et al., 2002; Wang et al., 2002). PNIPAAm can be modified with poly(ethylene glycol) (Turturro et al., 2011), chitosan (Chen & Cheng, 2006; Cho et al., 2004; Mu & Fang, 2008), collagen (Chen

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& Lee, 2008), hyaluronic acid (Ha et al., 2006; H.P. Tan et al., 2009), chondroitin sulfate (Varghese et al., 2008) or other polymers to adjust its gelling temperature and mechanical properties. This is to preserve the viability and phenotypic morphology, as well as improve proliferation, differentiation and extracellular matrix secretion of the cells entrapped within the hydrogel.

In this study, a thermo-sensitive comb-like polymer, aminated alginate-g-PNIPAAm (AAlg-g-PNIPAAm), was synthesized by coupling carboxylic end-capped PNIPAAm (PNIPAAm-COOH) to AAlg through amide bond linkages. The aim of this study is to investigate whether alginate hydrogel degradation could be controlled by PNIPAAm modification, and whether cell viability could be improved. In addition, the AAlg-g-PNIPAAm comb-like polymers were evaluated for their potential use as an injectable scaffold for tissue engineering.

2. Materials and methods

2.1. Synthesis of AAlg, PNIPAAm-COOH and AAlg-g-PNIPAAm

Aminated alginate (AAlg) was synthesized by a previously reported method on aminated hyaluronic acid with some modifications (Jia et al., 2006; H.P. Tan et al., 2009). Briefly, 0.5 g of sodium alginate was dissolved in 100 mL deionized H_2O to result in a 5 mg/mL solution. 10 g of adipic dihydrazide (ADH) was added to this alginate solution. 0.8 g of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 0.7 g 1-hydroxybenzotriazole hydrate (HOBt) were dissolved in dimethyl sulfoxide/H_2O (1:1, v/v, 5 mL each) and added to the reaction mixture. The pH of the solution was adjusted to 4.5 via 1 N HCl. This solution was stirred for 24 h and reacted at room temperature before being exhaustively dialyzed (molecular weight cut off, MWCO, 10,000) by deionized H_2O for 3 days. NaCl was then added to produce a 5% (w/v) solution and the AAlg was precipitated in ethanol. The AAlg was re-dissolved in deionized H_2O and re-dialyzed for 3 days to remove the salt. The purified product was freeze dried at −50 °C and kept at 4 °C. PNIPAAm-COOH was synthesized as described previously (Gao, Mohwald, & Shen, 2005; Mao et al., 2007). AAlg-g-PNIPAAm comb-like copolymers were synthesized by grafting PNIPAAm-COOH onto AAlg chains as shown in Fig. 1. 0.5 g of AAlg was dissolved in 100 mL deionized H_2O. To graft the PNIPAAm-COOH onto AAlg, PNIPAAm-COOH was dissolved in deionized H_2O and incubated with EDC at 4 °C for 48 h. The PNIPAAm-COOH/EDC amount was fixed with 5:1 ratio. The PNIPAAm-COOH solution was then added into the AAlg solution with varied weight ratios as follows: 1:1, 2:1 and 3:1, to fabricate three AAlg-g-PNIPAAm comb-like copolymer samples with different PNIPAAm grafting degrees. The mixed solution after agitation had a final pH value of 5.6. This solution was then incubated at room temperature for 24 h before dialysis (MWCO 25,000). The purified product was freeze dried at −50 °C and kept at 4 °C. ADH, EDC and HOBt were purchased from Aladdin Chemistry Co., Ltd. Other chemicals were purchased from Chemical Agents Co. Ltd., Beijing, China. For AAlg-g-PNIPAAm hydrogel formation, the AAlg-g-PNIPAAm was resolved in PBS at room temperature firstly, and then sitten for gel formation, it lasted 15 min at 37 °C.

2.2. Chemical characterization of AAlg, PNIPAAm-COOH and AAlg-g-PNIPAAm

The structure of AAlg and AAlg-g-PNIPAAm were determined by ATR-FTIR (Nicolet 560, USA) and ^{1}H NMR (300 MHz, JOEL, JNM-ECA300) spectra in comparison with alginate. FTIR spectra of all samples were acquired via accumulation of 256 scans with a resolution of 4 cm^{-1}. ^{1}H NMR spectra were measured at room temperature using D_2O as a solvent. The percentage of hydrazide group substitution in the AAlg polymer was quantified using the trinitro-benzene-sulfonic acid (TNBS) assay (Jens, 1979). The number-average molecular weight of PNIPAAm and the polydispersity index were measured by gel permeation chromatography (GPC, Waters Mode 515 HPLC pump, Milford, MA, USA) using tetrahydrofuran (THF) as a mobile phase. Monodisperse polystyrenes were used as standards. The remaining hydrazide groups of AAlg-g-PNIPAAm were also tested by the TNBS method to estimate substitution ratios of grafting PNIPAAm (molar percentage of alginate repeating units grafted by PNIPAAm).

2.3. Rheological analysis

AAlg-g-PNIPAAm comb-like copolymers were dissolved in deionized H_2O to form a solution with a concentration of 1 wt%. The rheological property of AAlg-g-PNIPAAm during the process of sol–gel transformation was tested by physica MCR300 Modular Compact Rheometer (Germany) with a constant shear rate (6.283 rad/s). Temperature sweep experiments from 25 to 40 °C were carried out at a heating rate of 3 °C/min.

2.4. In vitro degradation

Degradation of AAlg-g-PNIPAAm hydrogels was examined with respect to weight loss in PBS (pH = 7.4) with 100 U/mL penicillin and 0.1 mg/mL streptomycin. 3 wt% AAlg-g-PNIPAAm solution in PBS was placed in the mould with a size of Ø 1 cm × 1 cm. The solution formed cylindrical samples for the degradation test at 37 °C after 15 min. The 5 mL vial containing 4 mL PBS was used for an AAlg-g-PNIPAAm gel sample. The vials were incubated at 37 °C in a shaking incubator at 40 rpm for various time periods. Each week the buffer solution was replaced by a fresh one. At specified time intervals, hydrogels were quickly frozen at −40 °C, then lyophilized and weighed (W_t). The weight loss ratio was calculated as the following expression:

$$\text{Weight loss}\ \ (\%) = \frac{W_0 - W_t}{W_0} \times 100$$

Here W_0 and W_t are sample weights before and after degradation, respectively.

2.5. In vitro three-dimensional (3D) cell culture

Human bone mesenchymal stem cells (hBMSCs, purchased from Chinese Academy of Medical Sciences) were expanded in culture medium (89% DMEM containing 4500 mg/L D-glucose, 10% FBS, 1% P/S, Sigma). hBMSCs were encapsulated in AAlg-g-PNIPAAm hydrogels for 3D cell culture. Dry AAlg-g-PNIPAAm copolymer was sterilized under UV irradiation for 1 h and then dissolved in sterilized PBS at room temperature to obtain 3 wt% AAlg-g-PNIPAAm solution. The solution was exposed to UV irradiation for another 30 min. 5 mL AAlg-g-PNIPAAm solution was added into a sterilized centrifugal tube containing 1 mL hBMSC. The final cell density was 5×10^4/mL in the copolymer solution. Then the solutions containing cells were injected into a 12-well culture plate and incubated at 37 °C to form hydrogels (1 mL/well). After gelation, pre-warmed DMEM (37 °C) was added into each well and renewed daily. The living cells were stained with Calcein-AM (green) according to manufacturer's instructions. In brief, 1 mL Calcein-AM was added into each well of the 12-well culture plate at a concentration of 2 μmol/L. The

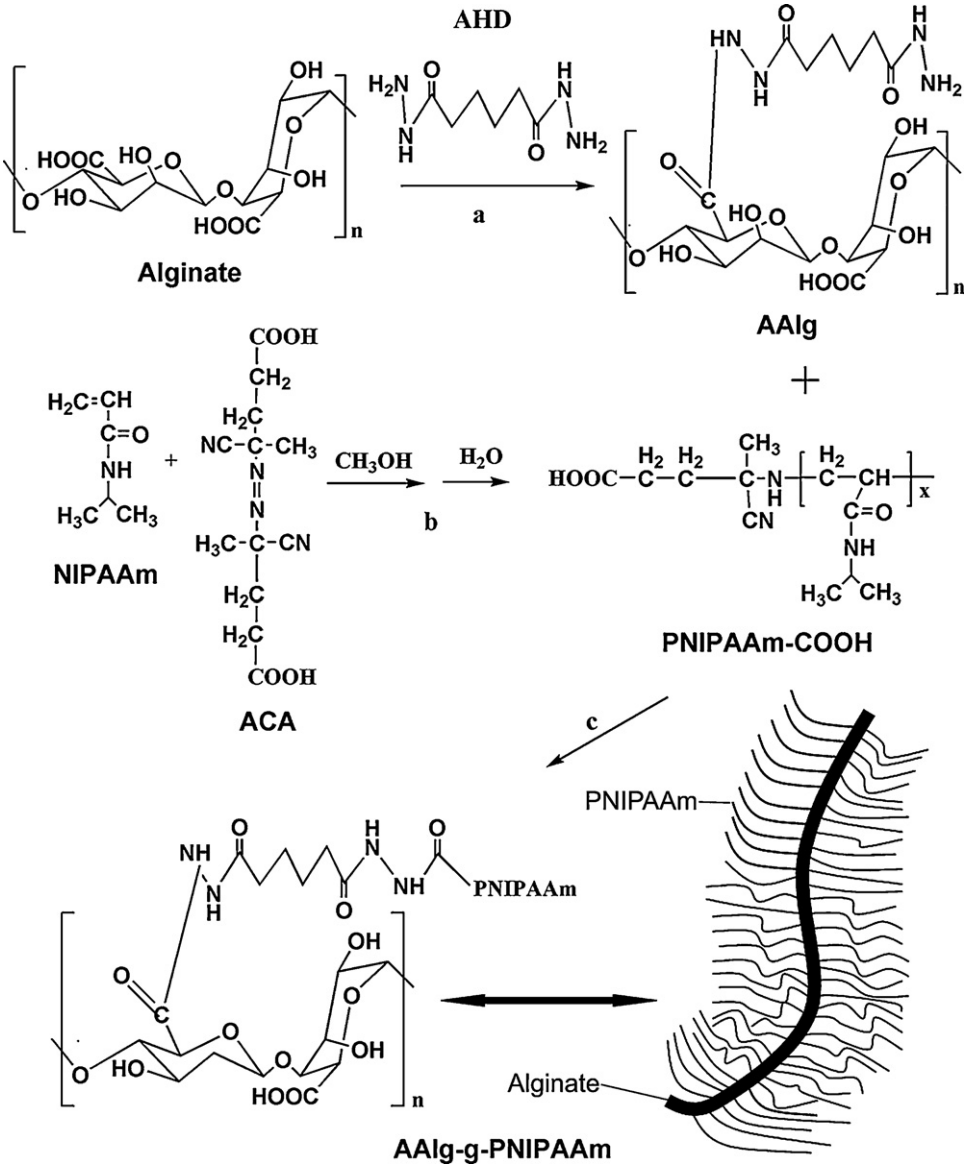

Fig. 1. Synthetic route and molecular structures of AAlg (a), PNIPAAm (b) and comb-like AAlg-g-PNIPAAm (c).

stained images were through a fluorescent microscope (Leica, Germany). The cell numbers in the AAlg-g-PNIPAAm copolymer hydrogels were determined quantitatively by measuring the amount of DNA using a CyQuant Cell Proliferation assay (Invitrogen, USA).

3. Results and discussion

3.1. Synthesis and characterization of AAlg-g-PNIPAAm comb-like copolymer

The AAlg-g-PNIPAAm comb-like copolymers were successfully synthesized (the synthesis routes are shown in Fig. 1), as determined by the FTIR and ^{1}H NMR spectra. Comparing FTIR spectra of alginate and AAlg (Fig. 2a and b), shows that despite the main characteristic peaks of alginate and AAlg being similar, the peak at 1320 cm^{-1} becomes weaker, and many weak peaks from 900 to 400 cm^{-1} in sodium alginate were replaced by a broad peak after modification of alginate with adipic dihydrazide (ADH), which indicates that ADH residues exist in the AAlg copolymer. The spectrum of AAlg-g-PNIPAAm (Fig. 2c) shows characteristic peaks of amide bands at 1650 and 1560 cm^{-1}, methyl groups in the isopropyl groups at 1380 cm^{-1}, and methylene groups of PNIPAAm backbone at 2980 cm^{-1} and 2940 cm^{-1}, which indicates that PNIPAAm residues exist in the AAlg-g-PNIPAAm copolymer. The structure of AAlg-g-PNIPAAm comb-like copolymers were further determined by the ^{1}H NMR spectra with evidence of proton peaks from sodium alginate residues (a'), ADH residues (b'–d') and NIPAAm residues (e'–h') (Fig. 3). Fig. 3a shows the hydroxyl proton peaks at 4.70 ppm of alginate (a'). Fig. 3b shows the ^{1}H NMR spectra of ADH residue proton peaks of AAlg (b'–d'). The peaks at 2.27 ppm (b') and 1.55 ppm (c') refer to methylene protons of the ADH residue of AAlg. In addition, a peak at 2.16 ppm was assigned to the acetamido protons (d') of AAlg. These results are similar to that of hyaluronic acid modified by ADH in previous reports (Jia et al., 2006; H.P. Tan et al., 2009). The ^{1}H NMR spectra of AAlg-g-PNIPAAm comb-like copolymers (Fig. 3c) present both the resonance peaks from ADH and PNIPAAm, demonstrating the success of the grafting reaction. As shown in spectra, the peaks at

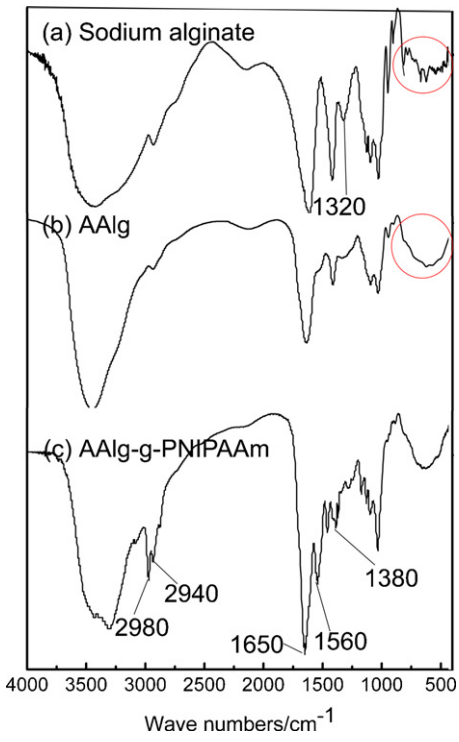

Fig. 2. FTIR spectra of sodium alginate (a), AAlg (b) and AAlg-g-PNIPAAm copolymer (c).

1.88, 1.33, 3.85 and 1.05 ppm are assigned to $-CH_2-$ (e'), –CH– (f'), –NH– (g') and $-CH_3$ (h') groups, respectively. These results are also similar to that of hyaluronic acid modified by PNIPAAm in a previous report (H.P. Tan et al., 2009). Besides structural analysis, the percentage of hydrazide group substitution in the AAlg polymer was quantified as 57% using the TNBS assay. The number-average molecular weight of PNIPAAm is 1.4×10^4 and polydispersity index is 1.4. The PNIPAAm grafting ratios of AAlg-g-PNIPAAm copolymers were 29%, 47% and 49% corresponding to weight ratios 1:1, 2:1 and 3:1 of PNIPAAm-COOH/AAlg, respectively. The PNIPAAm grafting ratios were not directly proportional to the increase in PNIPAAm-COOH/AAlg weight ratios, which might be contributed by grafting reactions limited by the stereo-hindrance effect.

3.2. Rheological test

The rheological properties of AAlg-g-PNIPAAm copolymer hydrogel were measured by monitoring the viscosity, storage modulus (G') and loss (G'') modulus as a function of temperature. The results of viscosity, G' and G'' as a function of temperature are shown in Fig. 4. In general, the viscosity of AAlg-g-PNIPAAm solutions can be seen to decrease with increased PNIPAAm grafting (Fig. 4a). The viscosity of AAlg-g-PNIPAAm with 29% PNIPAAm grafting is shown to be about five times that of AAlg-g-PNIPAAm with 47% or 49% PNIPAAm grafting. In addition, all AAlg-g-PNIPAAm solutions exhibit a decrease in viscosity as temperature was increased from 25 to 34 °C. From 34 to 36 °C, the viscosity of all AAlg-g-PNIPAAm solutions increased rapidly, as the aqueous solutions transformed to elastic hydrogels. The viscosity can be seen to quickly level off after 36 °C, which is an indication that the network structure of the AAlg-g-PNIPAAm hydrogels had formed completely (Fig. 4a). Similarly, the G' and G'' of AAlg-g-PNIPAAm with 29% PNIPAAm grafting were also higher (about six times) than that of AAlg-g-PNIPAAm with 47% or 49% PNIPAAm grafting. The G' and G'' of all AAlg-g-PNIPAAm solutions increased from 34 to 37 °C, but their changes were slower than that of viscosity. AAlg-g-PNIPAAm copolymer solutions transformed into stable hydrogels in situ at 37 °C, a phenomenon reflected by the levelling off of G' and G'' of all AAlg-g-PNIPAAm solutions after 37 °C. This sol–gel phase transition behavior is beneficial for cell entrapment to give a uniform distribution of cells within the gelated hydrogels (Chen & Cheng, 2006; H.P. Tan et al.,

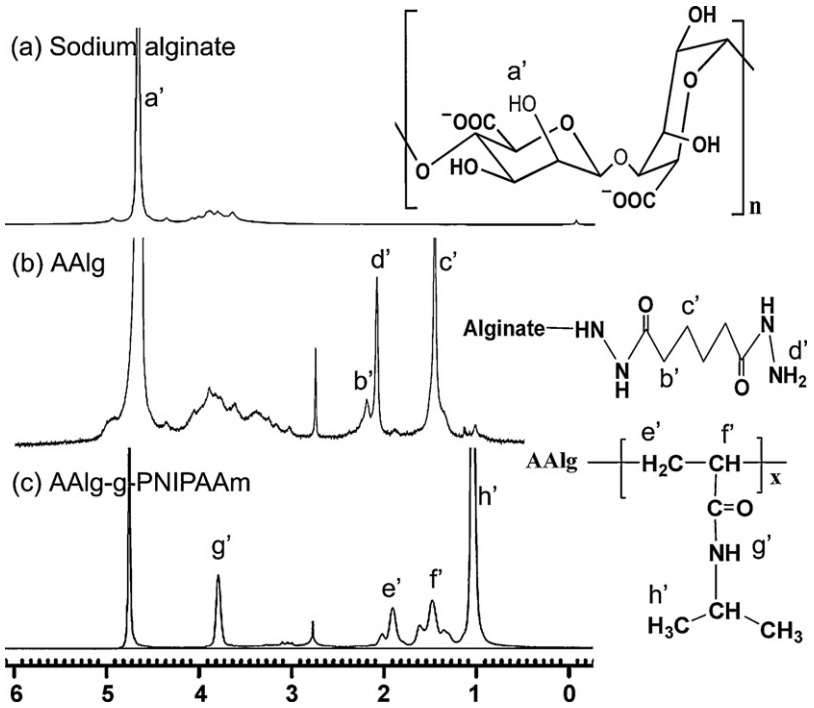

Fig. 3. ^{1}H NMR spectra of sodium alginate (a), AAlg (b) and AAlg-g-PNIPAAm (c).

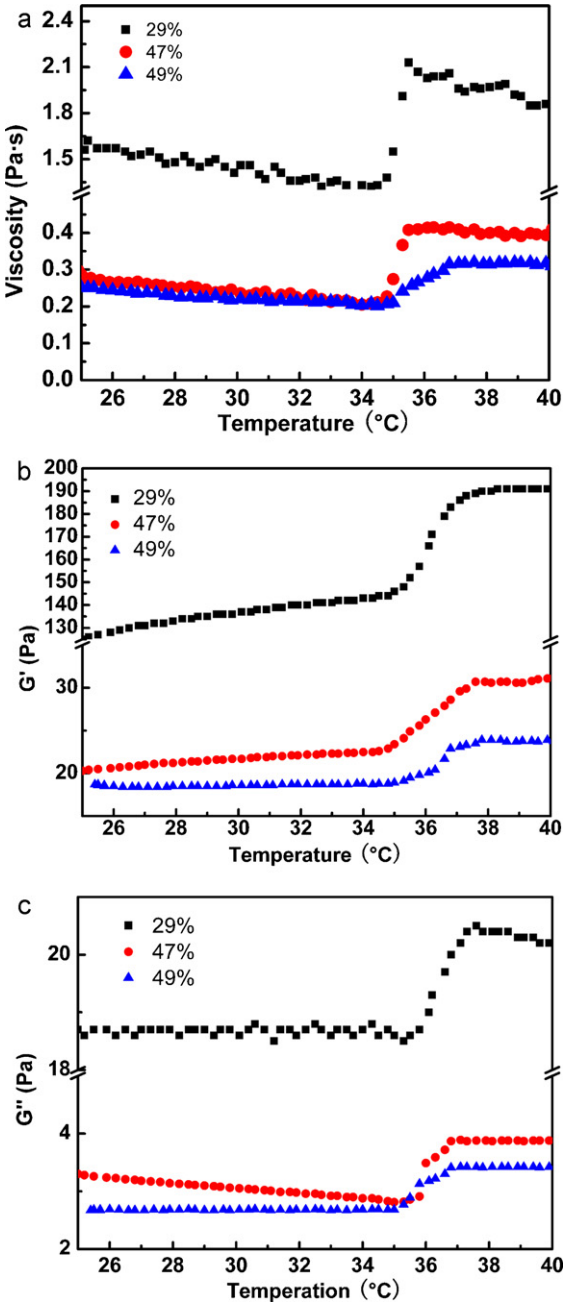

Fig. 4. (a) Viscosity curve of 1% AAlg-g-PNIPAAm copolymer with different grafting (29%, 47% and 49%) as a function of temperature. (b) Storage modulus (G') of 1% AAlg-g-PNIPAAm copolymer with different grafting. (c) Loss modulus (G'') of 1% AAlg-g-PNIPAAm copolymer with different grafting.

2009). It is also favourable for drug delivery, especially for proteins (Ha et al., 2006). The sharp changes in curves of viscosity, G' and G'' show that the LCSTs of all AAlg-g-PNIPAAm copolymer solutions are around 35 °C, which is higher than that of chitosan-g-PNIPAAm (around 30 °C) (Chen & Cheng, 2006), trimethyl chitosan-g-PNIPAAm (32 °C) (Mao et al., 2007) and aminated hyaluronic acid-g-PNIPAAm (around 30 °C) (H.P. Tan et al., 2009). Moreover, the grafted ratio of PNIPAAm demonstrates no influence on the phase transition behavior of AAlg-g-PNIPAAm copolymer solutions, which confirms that the LCST of AAlg-g-PNIPAAm is determined only by the hydrophilicity of the alginate macromolecular backbone.

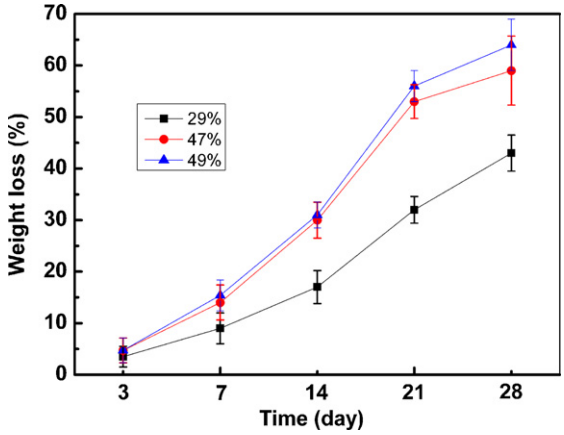

Fig. 5. Weight loss of AAlg-g-PNIPAAm copolymer hydrogels with different PNIPAAm grafting ratios (29%, 47% and 49%) in PBS at 37 °C. Values represent means ± standard deviation ($n = 7$).

3.3. *In vitro degradation of AAlg-g-PNIPAAm hydrogel*

Controlled degradability is a key factor for a biomaterial in tissue engineering and drug release. *In vitro* degradation study shows that AAlg-g-PNIPAAm hydrogels have a controllable degradation rate (Fig. 5). The degradation rates increase with increasing PNIPAAm grafting ratios. After four weeks, the weight loss of AAlg-g-PNIPAAm hydrogels with 29%, 47% and 49% grafting ratios are up to 43%, 59% and 64%, respectively. *In vitro* degradation study confirms that alginate hydrogel degradation can be controlled by PNIPAAm modification.

3.4. *Cytocompatibility of AAlg-g-PNIPAAm hydrogels*

The live encapsulated hBMSCs in AAlg-g-PNIPAAm hydrogels after 3D culture for 1 day and 7 days were stained by Calcein-AM and imaged by a fluorescent microscope (Fig. 6). Generally, the hBMSCs were uniformly distributed in all AAlg-g-PNIPAAm hydrogels. The encapsulated hBMSCs were shown to survive well and proliferate in copolymer hydrogels after 7 days culture. In addition, the cell numbers in the AAlg-g-PNIPAAm copolymer hydrogels were determined quantitatively by measuring the amount of DNA using a CyQuant Cell Proliferation assay. Fig. 7 shows the time course of changes in relative DNA content of hBMSCs in AAlg-PNIPAAm hydrogels. The DNA content in the three hydrogels progressively increased compared with initial one during 7 days culture. When comparing these three hydrogels, there is no significant difference of DNA contents after culture for 1, 3 and 7 days. However, the encapsulated hBMSCs in AAlg-PNIPAAm hydrogels have higher proliferation than the blank control group after 7 days culture ($p < 0.05$). In other words, the AAlg-g-PNIPAAm copolymer hydrogels are noncytotoxic and preserve the viability of the entrapped cells very well. From previous reports it is noted that the cytocompatibility of AAlg-g-PNIPAAm copolymer hydrogel is improved in comparison with alginate hydrogel when taken without any modification (Wang et al., 2003). This good cytocompatibility should be attributed to the microstructure and high water content of AAlg-g-PNIPAAm copolymer hydrogel, which is very similar to the extracellular matrix of natural tissue and is beneficial for cell surviving and proliferation.

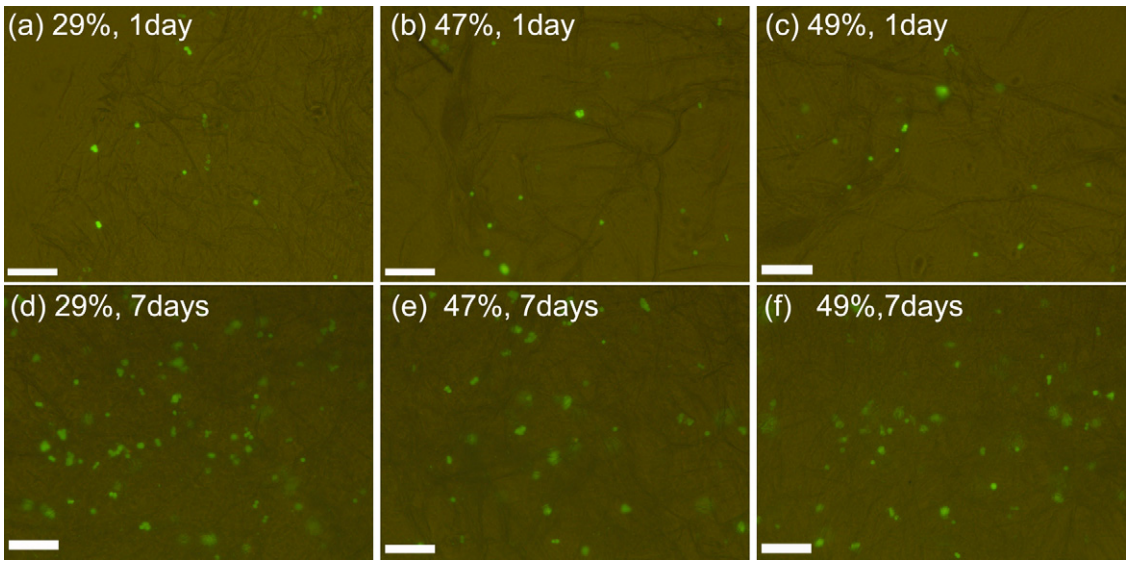

Fig. 6. Images showing encapsulated hBMSCs in AAlg-g-PNIPAAm copolymer hydrogels after 1 day (a–c) and 7 days (d–f) culture. The live cells were stained with Calcein-AM (green). The bar is 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

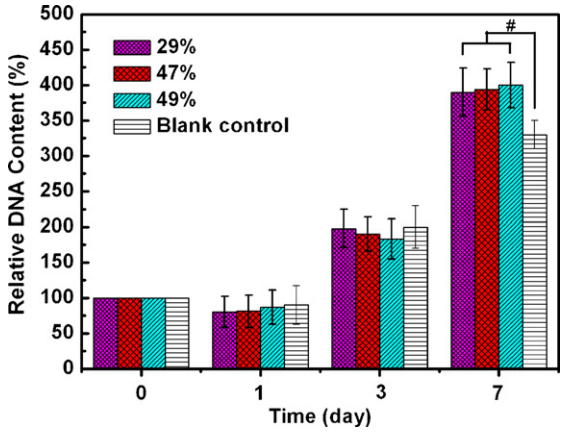

Fig. 7. DNA contents of encapsulated hBMSCs in AAlg-g-PNIPAAm hydrogels. Values represent means ± standard deviation ($n = 5$). $^{\#}p < 0.05$, blank control group vs. the other three groups.

4. Conclusion

A thermo-sensitive comb-like polymer with alginate as the backbone and PNIPAAm as pendant group has been synthesized successfully by grafting PNIPAAm-COOH with a carboxy end group onto aminated alginate through amide bond linkages. The copolymer exhibits thermo-sensitive sol–gel characteristics with LCST around 35 °C. The viscosity, G' and G'' decrease with increased PNIPAAm grafting. However, the grafted ratio of PNIPAAm demonstrates no influence on the LCST of AAlg-g-PNIPAAm copolymer solutions. *In vitro* degradation study confirmed that the degradation can be controlled by PNIPAAm modification. A preliminary *in vitro* cell culture study was performed using AAlg-g-PNIPAAm hydrogels as an injectable cell-carrier material to entrap hBMSCs. It was found the hydrogel not only preserved the viability of the entrapped cells but also stimulates the cell proliferation. These studies indicate that the thermo-sensitive AAlg-g-PNIPAAm hydrogel may have great potential in tissue engineering applications.

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