Carbohydrate Polymers 112 (2014) 480–485

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Effect of hyaluronic acid on the thermogelation and biocompatibility of its blends with methyl cellulose

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ARTICLE INFO

Article history:
Received 4 April 2014
Received in revised form 16 May 2014
Accepted 4 June 2014
Available online 17 June 2014

Keywords:
Hyaluronic acid
Methylcellulose
Drug delivery
Wound healing
Thermogelation
Polymeric blend

ABSTRACT

Aim of this work was to investigate the influence of hyaluronic acid (HA) molecular weight on the thermogelation and biocompatibility of its blends with methyl cellulose in view of a possible application in drug delivery and/or wound healing.

We found out that it was possible to obtain MC/HA blends showing a rheological behavior typical of a viscous solution at 20 °C and of a weak gel at 37 °C only when blending MC with low molecular weight HA. Moreover, the blends containing low molecular weight HA did not affect human foreskin fetal fibroblasts viability, proliferation and migration. On the contrary, the cell incubation with high molecular weight HA resulted in a marked and significant reduction of cell viability, compared to control cells. Finally, the optimized blends, in terms of rheological properties and biocompatibility, proved to be able to control and prolong bovine serum albumin release by a combined mechanism of platform dissolution and drug diffusion.

1. Introduction

Temperature-sensitive polymers hold great potential as drug delivery systems, mainly for their simple formulation, their ease of administration and the possibility to avoid the use of organic solvents (He, Kim, & Lee, 2008; Huynh et al., 2008; Klouda & Mikos, 2008; Mayol, Quaglia, Borzacchiello, Ambrosio, & La Rotonda, 2008; Shim, Yoo, Bae, & Lee, 2005). In particular, if the transition temperature is properly designed to be close to the physiologic temperature, these systems can undergo a sol-to-gel transition under physiological conditions without any additional external stimuli. Therefore, thermo-responsive gels can be simply administered into the body as a liquid drug dosage form and are able to generate *in situ* an entangled network structure which allows a sustained and controlled release of the active molecule(s). This is of special interest for the controlled release of biotechnological drugs, such as protein and peptides, which can be administered basically by parenteral routes due their poor biopharmaceutical profile. Moreover, these kind of matrices offer several advantages over systems shaped into their final form before implantation since, for example, injectable materials do not require a surgical procedure for placement and withdrawal if biodegradable. Finally, if they are used to fill a cavity of a wound or a defect, their flowing nature enables a good fit with a further advantage to release active macromolecules, such as grow factors, *in situ* with a controlled kinetic.

Cellulose derivatives are examples of thermosensitive polymers and, in particular, aqueous solutions of methylcellulose (MC), at low concentrations (1–10 wt%), are known to be liquid at low temperature but able to gel upon heating up to 40 and 50 °C (Desbrieres, Hirrien, & Rinaudo, 1998). It is possible to lower MC gelation temperature, up to the physiological one, by adding mono and divalent salts (Almeida, Rakesh, & Zhao, 2014) or by blending it with other polymers such as poly(acrylic acid) or polyethylen glycol, carboxymethyl cellulose and chitosan or hyaluronic acid (HA) (Caicco et al., 2013; Negim et al., 2014; Zhang et al., 2014). HA is a natural mucoadhesive polysaccharide, widely used in drug delivery and biomedical field, composed of alternating D-glucuronic acid (GlcA) and *N*-acetyl-D-glucosamine (GlcNAc) repeating units linked together via β-(1,4) and β-(1,3) glycosidic junctions (Borzacchiello, Mayol, Garskog, Dahlqvist, & Ambrosio, 2005; Borzacchiello et al., 2007; Mayol et al., 2014a; Borzacchiello, Mayol, Schiavinato, & Ambrosio, 2010). Polymeric blends represent an emerging class

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http://dx.doi.org/10.1016/j.carbpol.2014.06.020
0144-8617/

of biomaterials since they can combine the advantages of the polymers utilized. In particular, in the case of MC/HA blends, the thermosensitive properties of MC can be implemented with the well-known biocompatibility and mucoadhesivity properties of HA. In particular, a recent paper investigated the influence of polymers composition on MC/HA blend mechanical properties and cell survival (Caicco et al., 2013). However, in view of a possible application in the field of controlled macromolecules delivery, it is crucial the understanding of how the polymers interact each other leading to rheological synergism which determines their ability to control and sustain the release of macromolecular active molecules (Mayol et al., 2011).

In this context, the aim of this work was to investigate the influence of HA molecular weight (MW) on the rheological, thermogelation and biocompatibility properties of its blends with MC. In particular, we aimed to formulate a thermosensitive and biocompatible blend, with a phase transition temperature near to body temperature, able to release macromolecular active ingredients with a controlled kinetics. To this aim, here we analyzed the rheological properties of MC/HA blends, both with low molecular weight (LMW) and high molecular weight (HMW) HA, as a function of temperature. Furthermore, the human foreskin fetal fibroblasts (HFFF2) were used to exploit the biocompatibility of this system by cell viability, proliferation and migration assays. Finally, in order to explore the feasibility of these platforms for the sustained delivery of macromolecules, the optimized system was loaded with a model protein such as bovine serum albumin (BSA) and its release properties studied *in vitro*.

2. Materials and methods

2.1. Materials

Low molecular weight (150 kDa) HA was supplied by Fab (Abano Terme, Italy). High molecular weight HA (1×10^6 Da), MC, BSA, and phosphate buffer salts were purchased from Sigma (Milano, Italy).

2.2. Gel preparation

MC/HA-based formulations were prepared as follows. Half the solvent (PBS) was placed in a beaker at 0 °C, the other half heated up to boiling. The right amount of MC was dissolved in the hot solvent in which the cold solvent was then added. The obtained solution was placed in an ice bath under stirring. For a complete solubilization, the solution was left at 4 °C overnight, afterward HA at 1 and 2% w/v was added to MC solutions. For *in vitro* release tests, BSA was simply dispersed into the gel.

2.3. Rheological experiments

Rheological tests were performed by small-amplitude oscillatory shear experiments using a rotational rheometer (Malvern Kinexus) as previously reported (Maltese et al., 2006). Briefly, experiments were performed at 20 °C and 37 °C in the 0.1 to 10 Hz oscillation frequency range, and a strain amplitude at which linear viscoelastity is attained. The shear storage or elastic modulus (G') as well as the shear loss or viscous modulus (G'') were measured as a function of frequency.

2.4. Cell culture

The human fetal foreskin fibroblast (HFFF2) cell line was cultured at 37 °C in humidified 5% CO_2/95% air in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were plated in 24 culture wells at a density of 2.5×10^5 cells/ml per well and allowed to adhere upon the samples for 2 h.

2.5. Cell viability

The cell viability was determined by using 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) conversion assay (De Stefano et al., 2009). Briefly, 100 μl MTT (5 mg/ml in complete DMEM) were added and the cells were incubated for additional 3 h. After this time point the cells were lysed and the dark blue crystals solubilized with 500 μl of a solution containing 50% (v:v) *N*,*N*-dimethylformamide, 20% (w:v) SDS with an adjusted pH of 4.5. The optical density (OD) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCCC/340) equipped with a 620 nm filter. The cell viability in response to treatment with test compounds was calculated as % dead cells = 100 − (OD treated/OD control) × 100.

2.6. Cell proliferation assay

Proliferation was evaluated by incorporation of ^{3}H-thymidine (1 μCi/well) in HFFF-2 cells. Briefly, 2.5×10^5 cells were seeded in the wells upon the samples in a 24 well plate in the presence of ^{3}H-thymidine. The cells were then incubated for 24 h at 37 °C. After incubation, the cells were harvested, scraped in 1 M NaOH (100 μl/well) and collected on plastic miniature vials (PerkinElmer) automatic cell harvester prior to liquid scintillation counting (UltimaGold®, PerkinElmer). The effect on cell proliferation was expressed as count per minute per microgram of protein (CPM/μg protein) of incorporating ^{3}H-thymidine cells.

2.7. Cell migration: Scratch assay

Cells were seeded on the samples into 6 well cell culture plates as previously reported (Mayol et al., 2014b). Once at confluence, cells were serum-starved in medium containing 0.5% FBS over night, and then scratch injury was applied using a disposable pipette tip. After injury, the monolayer was gently washed with PBS, and the medium was replaced. Cell migration from the edges of the injured monolayer was examined and the area of population was measured 24 h after scratching by a computerized analysis system (LAS, Leica).

2.8. Statistics

Results are expressed as the means ± S.D. or S.E.M. of n experiments. Normally, the experiments were carried out in triplicate and three independent experiments were performed. Statistical significance was calculated by one-way analysis of variance (ANOVA) and Bonferroni-corrected *p*-value for multiple comparison test. The level of statistically significant difference was defined as $p < 0.05$.

2.9. In vitro release study

The optimized MC/HA blend was loaded with BSA and drug release profile was evaluated in phosphate buffered solution (PBS; 120 mM NaCl, 2.7 mM KCl, 10 mM phosphate salts; pH = 7.4), in a thermostatic bath at 37 °C in sink conditions as previously reported (Mayol et al., 2008). Briefly, drug delivery tests were carried out in glass cells where, at the bottom, a covered lid containing the polymer vehicle and drug, was placed. The cell was then filled with PBS and immersed in a thermostatic bath at 37 °C. When the temperature inside the cell reached 37 °C the lid cover was removed to allow the contact between the gel and the release medium. A magnetic stirrer into the cell provided a continuous agitation. At regular time intervals, 1 ml of solution was withdrawn from the cell and replaced

with fresh PBS. BSA was quantified by spectrophotometric assay ($\lambda = 279$ nm).

3. Results and discussion

3.1. Rheological study

The mechanical spectra, which is the dependence of both viscoelastic moduli (G' and G'') upon the frequency, of primary component solutions, containing only MC, LMWHA and HMWHA are reported in Figs. 1 and 2. All the primary component solutions display viscoelastic moduli that are frequency dependent since, by increasing the frequency, both moduli increase. Nevertheless, substantial differences among the rheological behavior of the three polymeric systems are observable. For MC and LMWHA solutions, $G'' > G'$ in the whole frequency range, indicating that these primary components behave as viscous solutions. In particular, MC solution at 2% w/v shows the same mechanical spectra at the two tested temperatures (37 °C and 20 °C). The HMWHA solutions, instead, at low frequency, shows a predominant viscous character ($G'' > G'$) but, by increasing the frequency, the elastic modulus grows faster then the viscous one so that G' curve crosses G'' curve at a frequency called cross-over frequency (ω_c), after which the elastic modulus prevails on the viscous one and the system shows a predominant elastic character. This rheological behavior is typical of an entangled network (Ambrosio, Borzacchiello, Netti, & Nicolais, 1999).

In Fig. 3, the mechanical spectra of blends made up of MC (2% w/v) and LMWHA (at 1 and 2% w/v) at the two tested temperatures (37 °C and 20 °C) are reported. The rheological behavior of LMWHA/MC blends is temperature dependent. In particular, LMWHA/MC blends assume a typical behavior of a viscous fluid or entangled solutions at 20 °C while, at 37 °C, of a weak gel since $G' > G''$ in all the frequency range examined with both the viscoelastic moduli less dependent upon frequency. Moreover, the presence of LMWHA into MC solutions led to an increase of both the viscoelastic moduli which was not proportional to the HA content. In particular, at 37 °C and at the frequency of 1 Hz, G' of MC is 7.2 Pa while G' of MC/LMWHA is 40 Pa and 30 Pa from blends containing, respectively, 1 and 2% w/v of LMWHA. By further increasing the concentration of both the polymers into the blend the shear zero

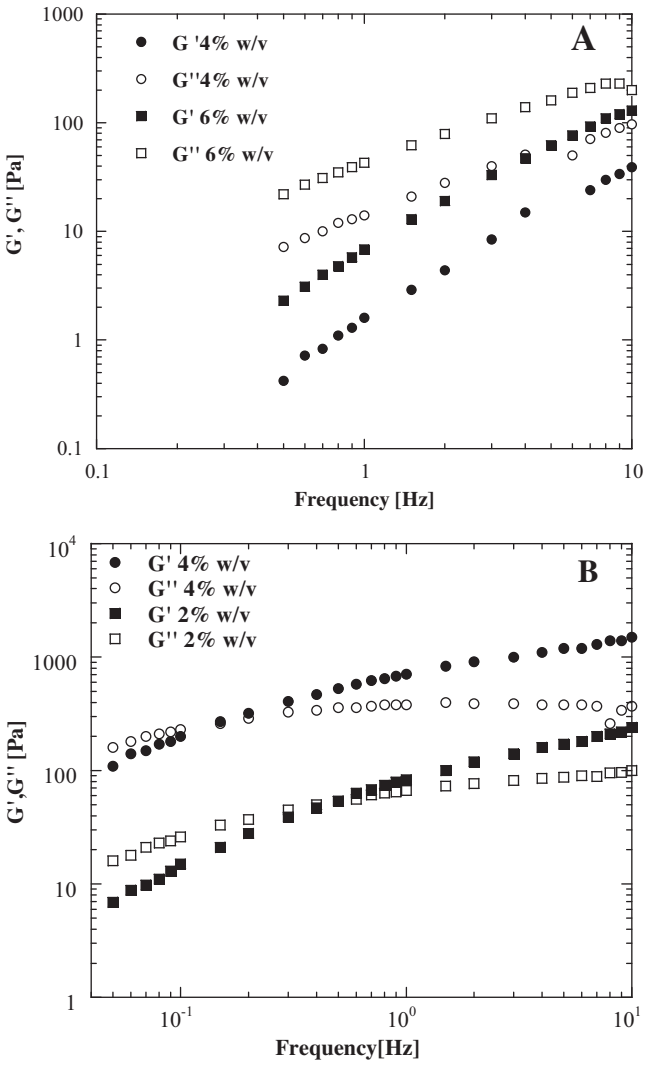

Fig. 2. Mechanical spectra of LMWHA at 4 and 6% w/v (A) and HMWHA at 2 and 4% w/v (B).

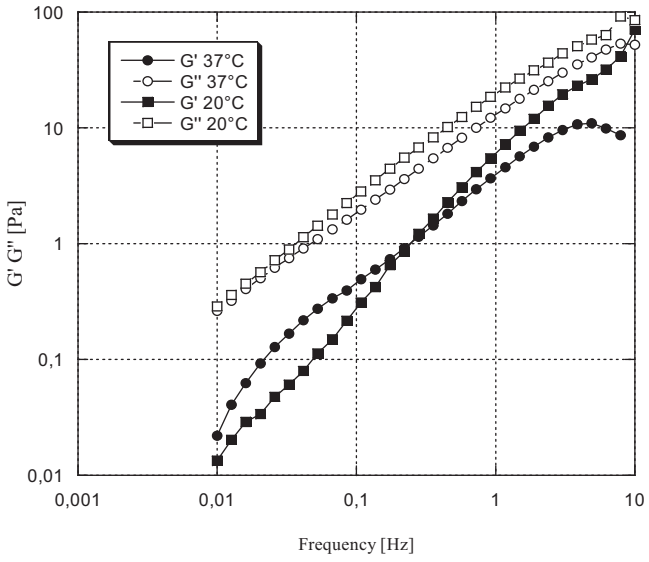

Fig. 1. Mechanical spectra of MC at 2% w/v at 37 °C and 20 °C.

viscosity of the system increases too much thus compromising its injectability.

The gelation process of MC solutions is primarily caused by the hydrophobic interaction between molecules containing methoxy substitution (Desbrieres et al., 1998). In particular, at low temperatures, the macromolecules are hydrated, and there is little polymer–polymer interaction other than simple entanglements. As the temperature is raised, the polymers gradually lose their water of hydration and this can favor polymer–polymer associations which leads to the gelification of the system. These phase transition temperatures can be lowered by chemical or physical modifications. For example, NaCl decreases the transition temperature of methylcellulose solutions to 32–34 °C (Ruel-Gariepy & Leroux, 2004). The effect of LMWHA on the thermogelation of its blend with MC can be explained as follows. In solution, HA displays an extraordinary capability to retain water and behaves as an expanded random coil occupying a large hydrodynamic volume (Ambrosio et al., 1999). As a consequence, the presence of HA in solutions can accelerate the desolvation of MC molecules thus reducing its transition temperature. The same effect was already observed in blends of HA and other thermosensitive polymers such as poloxamers (Mayol et al., 2008). In Fig. 4, the mechanical spectra of MC/HMWHA blends is reported. As it can be observed, the MC/HMWHA blends

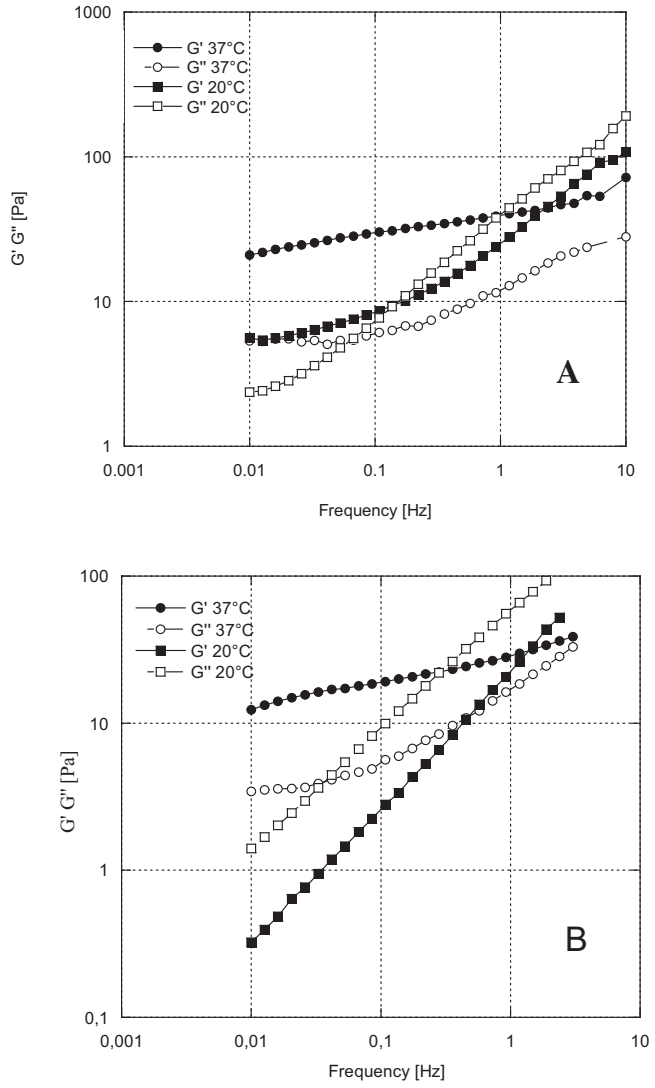

Fig. 3. Mechanical spectra of MC/LMWHA blends at the two tested temperature (37 °C and 20 °C) and composition: (A) MC 2% w/v + LMWHA 1% w/v and (B) MC 2% w/v + LMWHA 2% w/v.

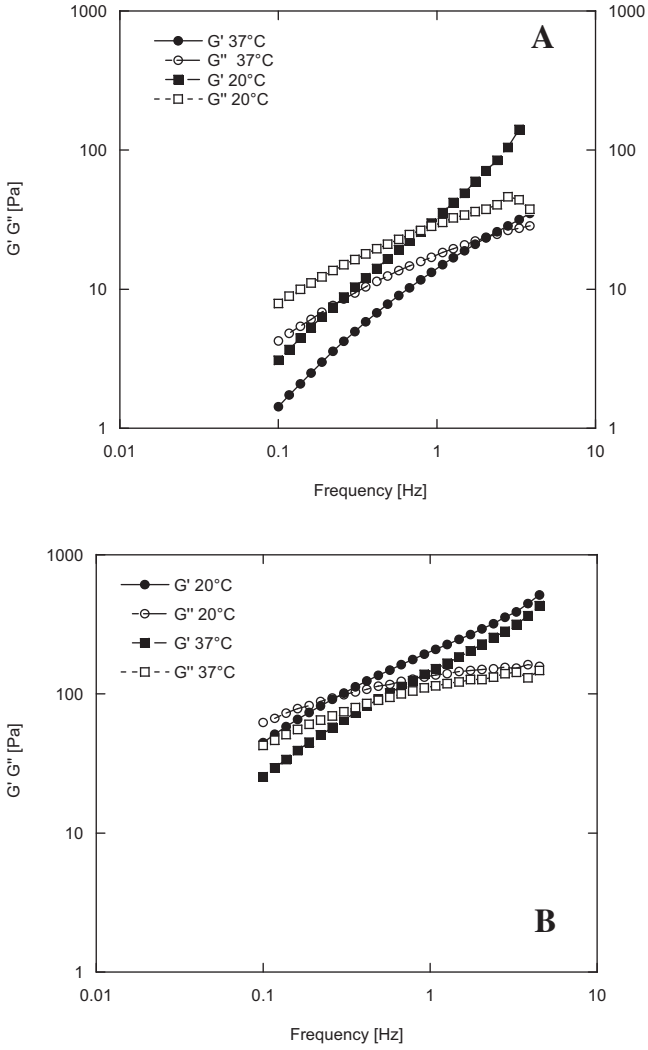

Fig. 4. Mechanical spectra of MC/HMWHA blends at the two tested temperature (37 °C and 20 °C) and composition: (A) MC 2% w/v + HMWHA 1% w/v and (B) MC 2% w/v + HMWHA 2% w/v.

behaves as an entangled network solution at all the concentration tested and at both the temperature examined, *i.e.* body and room temperature. Moreover, from a quantitative point of view, the viscoelastic moduli values are similar to those of HMWHA solutions (Fig. 2). These results suggest that, when the length of HA polymer chains became too high, or the HA random coil at rest is too large, the interactions between the methoxy substitution of MC chains are obstructed thus preventing the gelation of the system.

3.2. Biocompatibility studies

To evaluate cell viability and proliferation, the MC formulations were tested on HFFF2 cells by MTT and ^{3}H methyl thymidine assay, respectively. Incubation of HFFF2 cells with MC (1, 2 or 3%) for 24 h did not modify cell viability (Fig. 5A), cell proliferation (Fig. 5B) neither cell migration (Fig. 5C) when compared with untreated (control) cells. These observations demonstrate that MC is not toxic and suggest that it is a useful platform for the preparation of an inert bioactive gel. We decided to continue our studies on MC gel at 2% w/v, being this concentration a good compromise between biocompatibility and rheological properties since, as above evidenced, the polymer blends of MC at 2% w/v with LMWHA showed a temperature dependent behavior.

Therefore, HA formulations were tested on HFFF2 cells by MTT assay. The incubation of HFFF2 cells with LMWHA (1, 2 or 3%) for 24 h did not modify cell viability when compared with untreated cells. Instead, the cell incubation with HMWHA at both HA concentrations (2 and 3%) resulted in a marked and significant reduction of cell viability, compared to control cells (Fig. 6).

Therefore, this study has directed attention to the preparation of MC/LMWHA blends. The effects of MC/LMWHA blends on cell viability, proliferation and migration are shown in Fig. 7. Incubation of HFFF2 cells with MC (2%)/LMWHA (1%) for 24 h did not modify cell viability (Fig. 7A), cell proliferation (Fig. 7B) neither cell migration (Fig. 7C) when compared with untreated cells. In contrast, the cell incubation with MC (2%)/LMWHA (2%) resulted in a marked and significant reduction of cell viability, proliferation and migration compared to control cells (Fig. 7A–C). Altogether these data suggest that the MC (2%)/LMWHA (1%) gel is the best platform for the inert delivery of bioactive molecules.

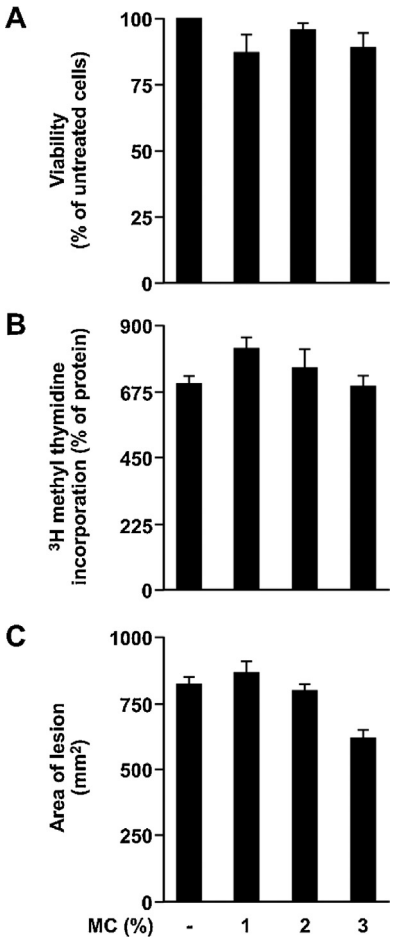

Fig. 5. Effect of MC on HFFF2 cells. HFFF2 cells were incubated upon MC formulations (1, 2 or 3% w/v) for 24 h. Thereafter, the cell viability (A), cell proliferation (B) and cell migration (C) were determined as described in Section 2. Data are expressed as mean ± S.E.M. of three separate experiments in triplicate.

3.3. *In vitro* release studies

Results from both rheological and biocompatibility analysis directed the *in vitro* drug release study on MC/LMWHA blend, with 2% w/v of MC and 1% w/v of LMWHA, being both thermosensitive and biocompatible. As for the drug choice, BSA was loaded into the MC/LMHA blend as a model protein in order to explore their capability to control and sustain the release of macromolecular active molecules, such as proteins and/or growth factors.

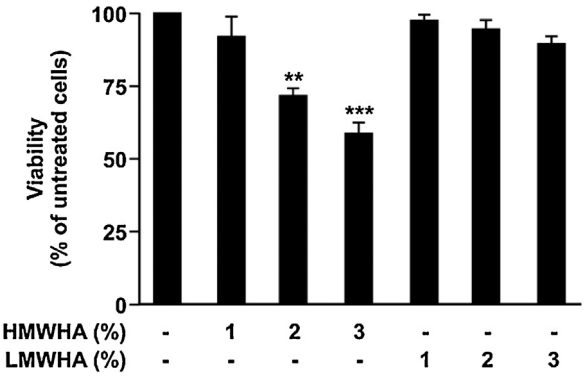

Fig. 6. Effect of HA on HFFF2 cell viability. HFFF2 cells were incubated upon LMWHA or HMWHA formulations (1, 2 or 3% w/v) for 24 h. The cell viability was determined by MTT assay as described in Section 2. Data are expressed as mean ± S.E.M. of three separate experiments in triplicate (** $p < 0.01$ and *** $p < 0.001$ *vs.* control cells).

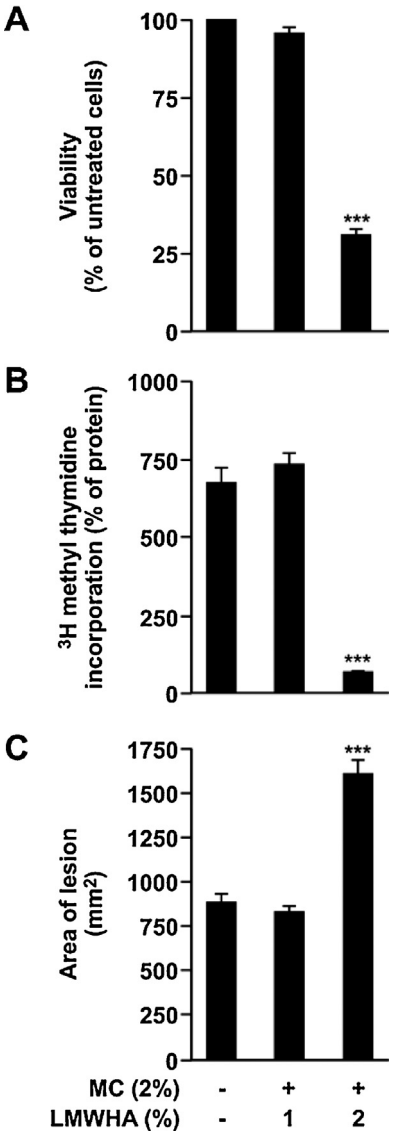

Fig. 7. Effect of MC/LMWHA blends on HFFF2 cells. HFFF2 cells were incubated upon the indicated blends for 24 h. Thereafter, the cell viability (A), cell proliferation (B) and cell migration (C) were determined as described in Section 2. Data are expressed as mean ± S.E.M. of three separate experiments in triplicate (*** $p < 0.001$ *vs.* control cells).

The *in vitro* release kinetics of BSA from the optimized MC/LMWHA blend is reported in Fig. 8. The tested platform showed a slow protein release rate, characterized by a three-phasic profile. In the first hours, an initial fast release, the so called *burst* release, of about 20% was observed. A second release phase was characterized by a slower release rate for about 3 h, followed by a further slowing down of the release profile for about 65 h. This three-phasic release profile should be the result of different typical mechanisms of drug release from hydrogels (Peppas, Bures, Leobandung, & Ichikawa, 2000). The *burst* release observed in the first hours of release could be due to the drug closer to the surface of the polymeric device. In the second phase, diffusion of BSA from the polymeric matrix could become predominant; finally, the release rate in the third release phase, could interest BSA deeply entrapped into the polymeric matrix for which the slow polymeric dissolution of the matrix could control the drug release. Due to the relatively short release period, we suggest this polymeric platform for application in which

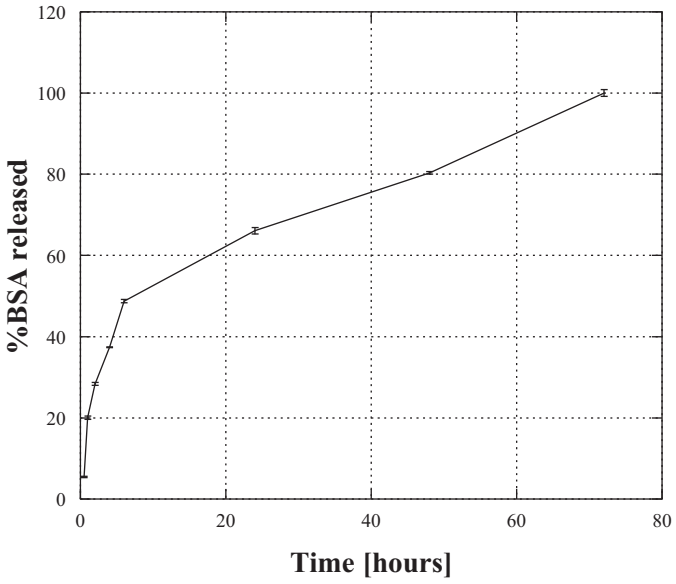

Fig. 8. Release kinetics of BSA from the optimized MC/LMWHA blend.

medication should be replaced with a frequency of 2–3 days, such as drug delivery into a wound. The biocompatibility of this blend on fibroblasts can support this proposed application.

4. Conclusions

The aim of the present study was to exploit the effect of HA molecular weight on the thermogelation and biocompatibility properties of MC in view of a possible application of this blends for drug delivery and/or wound healing.

Biocompatibility tests revealed that the MC/LMWHA blends were not toxic on human fibroblasts, differently from the ones containing HMWHA. Rheological analysis reveled that the MC/LMWHA blends possessed a thermo-sensitive rheological behavior. In particular, the optimized formulation can flow as a viscous fluid at 20 °C while, at 37 °C, can behave as a weak gel. On the contrary, the presence of HMWHA into the blend with MC, in the same concentration range, prevent the thermogelation process. The potential of these thermosensitive gels as polymeric platform for the controlled release of high molecular weight molecules, such as protein, has been demonstrated *in vitro*.

Acknowledgment

This work was supported by Italian “Ministero della Salute” (Bando Giovani Ricercatori RF 2007 (GR-2007-687941) “Ricerca e sviluppo di nuovi gel bioattivi per la medicazione di piaghe da decubito”).

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