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Evaluation of *in situ* injectable hydrogels as controlled release device for ANXA1 derived peptide in wound healing

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ABSTRACT

In this paper, for the first time, hydrogels containing Annexin A1 N-terminal derived peptide, Ac2-26, as a novel dressing were successfully developed for dermal wound repair application. High mannuronic (M) content alginate and low molecular weight chitosan have been used as hydrogel carrier. Peptide recovery analyses, FTIR studies and molecular modelling highlighted chemical interactions between peptide and hydrogel polymers. Ac2-26 resulted entrapped into chitosan hydrogel matrix that prevented its release, whereas such interaction in alginate hydrogel slowed down peptide diffusion enabling its sustained release till 72 h. *In vivo* wound healing studies conducted on mice dorsal wounds indicate that after the 9th day of post wounding Ac2-26/alginate hydrogels could significantly accelerate wound healing, with complete closure of the wound on day 14th. Therefore, these results suggest that the developed of Ac2-26 high M content alginate hydrogel could be a promising wound dressing with potential application in dermal wound healing.

1. Introduction

Wound healing is an ordered process including a variety of cellular and matrix components that results in contraction and closure of the wound tissue as well as in restoration of the barrier functions (Werner, Krieg, & Smola, 2007). However, in some cases, wounds fail to close as in diabetic, venous and pressure ulcers, representing a significant burden to patients, as well as health, economic and social problem in the last decades (Sen et al., 2009). The discovery of growth factors (GFs) and their mechanisms of action was a strong stimuli to the design of locally controlled release formulations loaded with different GF molecules (EGF, VEGF, bFGF, GM-CSF, PDGF-BB). Despite the encouraging results from various clinical trials only PDGF-BB has been approved for its use in diabetic ulcers as a gel formulation (Hirshberg, Coleman, Marchant, & Rees, 2001; LeGrand, 1998). However, it showed a modest efficacy and phase IV investigations highlighted significant side effects in patient extensively treated with PDGF (Margolis, Crombleholme, & Herlyn, 2000); therefore, other growth-factors and new formulations must be tested and developed (Chéret, Lebonvallet, Carré, Misery, & Le Gall-Ianotto, 2013).

Annexin A1 (ANXA1, lipocortin-1) is the first characterized member of the annexin superfamily of proteins, able to bind (*i.e.*, to annex) to cellular membranes in a Ca^{2+}-dependent way. ANXA1 has been involved in a broad range of molecular and cellular processes, including anti-inflammatory signalling, kinase activities in signal transduction, maintenance of cytoskeleton and extracellular matrix integrity, tissue growth, apoptosis and differentiation (Gerke & Moss, 2002). ANXA1 N-terminal derived peptide Ac2-26 has been used as an ANXA1 surrogate in view of its ability to replicate the anti-inflammatory effects of the parent protein (Perretti & Dalli, 2009). Ac2-26 can activate all three human formyl peptide receptors, promoting calcium fluxes, and cell motility (Katanaev, 2001). Such an effect may be part of a reparatory process as demonstrated with intestinal epithelial cells, skeletal muscle myoblasts and WS1 human fibroblasts (Babbin et al., 2006; Bizzarro, Belvedere, Dal Piaz, Parente, & Petrella, 2012a; Bizzarro et al., 2012b) where addition of the Ac2-26 peptide engaged FPRs to activate the cytoskeletal machinery amplifying the pathway downstream FPR activation, contributing to actin polymerization and then favouring cell migration and tissue repair.

Natural and biocompatible hydrogels have applicability in wound dressing because of their high water content which should maintain appropriate moisture at the wound bed (De Cicco, Porta, Sansone, Aquino, & Del Gaudio, 2014a; Porta et al., 2010). In addition they are transparent allowing the monitoring of the healing

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process. The ideal dressing material should have proper adherence to the wound site, good exudate absorbance, easy application and removal and must be able to delivery and control drug release.

Alginates and chitosans have been used as wound dressing due to their adhesive properties as well as antimicrobial activity and stimulation of healing (A°gren, 1996; Murakami et al., 2010; White, 2011). Alginates are composed by a mixture of two monomeric units, guluronic acid (G) and mannuronic acid (M). High M content alginates may induce cytokine production by human monocytes, a process very useful in chronic wound healing (Thomas, Harding, & Moore, 2000). Low molecular weight (LMW) chitosan is also able to induce macrophages activation by interaction of the acetylated residues with mannose receptors (Porporatto, Bianco, Riera, & Correa, 2003). This action as well as collagenase activity can be enhanced by chitosan oligomers and monomers easy produced by enzymatic degradation of LMW chitosan (Minagawa, Okamura, Shigemasa, Minami, & Okamoto, 2007).

In the present study, we develop a hydrogel based either on high M content alginate or LMW chitosan loaded with Ac2-26 with the aim to obtain a topical controlled release formulation with enhanced wound healing activity. Investigation on gel consistency, swelling, fluid uptake properties as well as stability studies on peptide loaded hydrogels and peptide release behaviours have been conducted. *In vivo* wound healing activity of the most promising formulations has been evaluated on mice.

2. Materials and methods

A solution (1 mM) of ANXA1 N-terminal peptide Ac2-26, having the following sequence: Ac-Ala-Met-Val-Ser-Glu-Phe-Leu-Lys-Gln-Ala-Trp-Phe-Ile-Glu-Asn-Glu-Glu-Gln-Glu-Tyr-Val-Gln Thr-Val-Lys, was purchased from Tocris Bioscience (Bristol, UK). Sodium alginate from brown algae (1% viscosity 35 mPa s; mannuronic acid content 70%) was purchased from Carlo Erba reagents (Milan, Italy). Chitosan low molecular weight (1% viscosity in acetic acid 20–80 mPa s; 75–85% deacetylated) was purchased from Sigma Aldrich (Milan, Italy). All other chemicals and reagents were obtained from Sigma Aldrich (Milan, Italy) and used as supplied.

2.1. Hydrogels preparation

Alginate powder was dispersed in distilled water under vigorous stirring (1500 rpm) for 15 min then the solution was left under gentle stirring (100 rpm) for 2 h. Finally polymer solutions were left motionless for 4 h to remove air bubbles (Gaudio, Russo, Lauro, Colombo, & Aquino, 2009).

Chitosan gels were prepared using the same procedure dissolving chitosan powder in 0.1 M acetic acid buffer solution then pH was adjusted to 6.3 using 0.1 M NaOH aqueous solution.

Ac2-26 peptide loaded gels were prepared by mixing in an orbital shaker (Vortex Genius 3, IKA-Werke GmbH & Co., Staufen, Germany) a precise volume of either high M alginate or LMW chitosan solution by means of an Hamilton syringe (Hamilton AG, Bonaduz, Switzerland) and a precise volume of a peptide stock solution by means of a Gilson pipet (Pipetman Classic, Gilson, Inc., Middleton, WI, USA) in order to obtain a final peptide concentration of 100 nM, 500 nM and 1 μM in either alginate solution at 2.5% and 5.0% (w/w) or chitosan solution at 3.5% and 6.5% (w/w).

2.2. Peptide recovery analyses

The Ac2-26 recovery from different hydrogels was evaluated by LC/MS analyses, in that aims eight different samples were prepared loading two different peptide concentrations (100 nM and 1 μM), in either alginate solution at 2.5% and 5.0% (w/w) or chitosan solution at 3.5% and 6.5% (w/w). Following 6 h of incubation, 1 ml of each sample was pre-purified on a ChromabondHR-X SPE cartridge (Macherey-Nagel, Düren, Germany) using water and 70% acetonitrile as washing and elution solution, respectively. LC/MS analyses were performed on a LTQ XL instrument (ThermoScientific) equipped by an ESI ion source and a hybrid quadrupole/linear trap analyzer and coupled with an Accela 600 HPLC system. Chromatography was performed on an Aeris C18 (2.0 × 150-millimeter i.d., 3 μm) reversed-phase column, using a mobile phases A (0.1% formic acid in water, v/v) and B (0.1% formic acid in acetonitrile, v/v), using a linear gradient increase from 25 to 70% B in 30 min. The flow rate was 200 μl/min. Mass spectra were acquired in positive ion mode over the m/z range from 700 to 1600. In order to measure the amount of AC2-26 in each sample, a selective chromatogram for this species was obtained, selecting ions at m/z 773.37 $[M+4H]^{4+}$, 1030.82 $[M+3H]^{3+}$, and 1545.73 $[M+2H]^{2+}$.

2.3. Hydrogels physico-chemical properties

Infrared analysis was performed using a FTIR spectrophotometer (IRAffinity-1S, Shimadzu Corporation, Kyoto, Japan) equipped with a MIRacle ATR accessory with ZnSe crystal plate. The samples were directly spread over the crystal plate and analysed using 256 scans with a 1 cm^{-1} resolution step. Each experiment was carried out in triplicate, and results averaged.

Each polymer solution was subjected to a complete rheological characterization using a rotational rheometer (Bohlin CS Rheometer, Bohlin Instrument Division, Metrics Group Ltd., Cirencester, UK). A cone plate combination (CP 4/40) was used as measuring system. All measurements were carried out at 37 °C, after a rest time of 3 min. Zero shear rate viscosity, η_0, was obtained after application of 50 s^{-1} shear rate for 1 min. Dynamic oscillatory tests were performed in the linear viscoelastic range. The viscoelastic parameters storage modulus (G') and loss modulus (G'') were measured at frequencies ranging between 0.1 and 5 Hz (Aquino et al., 2012; Gaudio, Colombo, Colombo, Russo, & Sonvico, 2005). Experiments were done in triplicate.

Fluid loss of all formulations was evaluated over time as the ratio between fluid content of the gel at a specific time point and a carefully weighted amount of gel after preparation. Briefly, 250 μl of formulation was spread over a previously weighted filter paper disc. The membrane was in contact with a donor compartment filled with 100 μl of simulated wound fluid (SWF) consisting in 50% foetal calf serum (Sigma Aldrich, Milan, Italy) and 50% maximum recovery diluent (Sigma Aldrich, Milan, Italy), composed by 0.1% (w/v) peptone, peptic digest of animal tissue, and 0.9% (w/v) sodium chloride) (Aquino et al., 2013; Bowler et al., 2012). At regular intervals of time, the weight of the gel was measured. Fluid loss percentage was associated to weight loss and calculated by the following equation:

$$\text{Fluid}\ \ (\%) = \frac{W_t}{W_0} \times 100$$

where W_0 is the initial weight and W_t is the weight at the specific time point. All experiments were performed at least in triplicate.

Molecular dynamics simulations were performed using YASARA 12.7.1650 with the NPT ensemble at 310 K and 1 atm. The YAMBER3 force field was used. All simulations ran with a 1.25 fs time step for intramolecular forces and a 2.50 fs time step for intermolecular forces. The equilibration period was 20 ns.

2.4. Peptide release studies

AC2-26 release assays were performed by means of slide-a-lyzer mini dialysis units, 10 K molecular weight cut off (Thermo

Table 1
Composition, zero shear rate and mid-value dehydration data of both high mannuronic content alginate and low molecular weight chitosan Ac2-26 loaded hydrogels. Each value represents the mean ± S.D. ($n \geq 3$).

Sample code	Alginate concentration % (w/w)	Chitosan concentration % (w/w)	Ac2-26 concentration (μM)	Zero shear viscosity (η_0) (Pa s)	Fluid loss mid-value (h)
ACA25-100	2.5	–	0.1	4.21×10^2	52
ACA25-500			0.5		
ACA25-1000			1.0		
ACA50-100	5.0	–	0.1	9.50×10^2	58
ACA50-500			0.5		
ACA50-1000			1.0		
ACC35-100	–	3.5	0.1	4.10×10^2	36
ACC35-500			0.5		
ACC35-1000			1.0		
ACC65-100	–	6.5	0.1	9.48×10^2	48
ACC65-500			0.5		
ACC65-1000			1.0		

Fisher Scientific Inc., Waltham, MA, USA) with an exposed surface area of 0.33 cm^2. The dialysis cup was filled with 200 μl of AC2-26 loaded gel. Experiments were conducted in a cylindrical vial filled with 3 ml of simulated wound fluid (SWF) thermostated at 37 °C and magnetically stirred at 200 rpm by a teflon-coated stirring bar placed in the receptor compartment. The receptor solution, 100 μl aliquots, were sampled at specific time points and analysed by LC-MS for the determination of AC2-26 permeated through the dialysis membrane, as previously described (see 2.2).

2.5. In vivo wound healing test

C57BL/6 mice were obtained from Harlan Laboratories (Indianapolis, IN, USA) and housed 4 per cage prior and 1 per cage during experiments. On the day of surgery, the hair on the back of each mouse was clipped, and the skin was wiped with ethanol. C57BL/6 mice, 10 weeks of age, were anesthetized with isoflurane prior to the wound-creating surgery.

Full-thickness excision wounds (1 cm × 1 cm) were created by marking the area of the wound in the shaved mid-back with a fine marker and a ruler, lifting the skin with a pair of a forceps and excising the full-thickness skin along the lines with a pair of surgical scissors. Immediately after the surgery on day 0, the wounds were topically treated with 100 μl of either 5.0% alginate gel (placebo) or the same gel containing Ac2-26 peptide. Penicillin (5 mg per mouse) was injected intramuscularly to prevent infections.

Sixty mice were randomly divided into five groups: (1) control group; (2) 5.0% alginate gel; (3) Ac2-26 100 nM/alginate hydrogel group; (4) Ac2-26 500 nM/alginate hydrogel group; (5) Ac2-26 1 μM/alginate hydrogel group, twelve mice for each group. The control group was treated with 0.5 ml saline solution topically; the blank 5.0% alginate hydrogel group was treated with 0.5 ml of 2.5% alginate hydrogel administered topically; the Ac2-26 loaded 5.0% alginate hydrogel group was treated with 0.5 ml of Ac2-26 loaded 2.5% alginate hydrogel (containing 100 nM, 500 nM or 1 μM of Ac2-26 peptide) administered topically.

On 3th, 6th, 9th and 14th day of post-wounding, three mice from each group were sacrificed and the size of wound area was recorded.

The percentage of wound healing was based on changes in the same wound on the same mouse for indicated time points, instead of changes among different mice. Thus, the calculated mean for the wound healing (percentage) among the 3 mice in a group was independently obtained, and the statistics of 3 groups of 3 independent experiments were calculated. We defined and presented the healing in 2 ways: (a) the open area of the healing wound on a given day/the open area of the original wound × 100 for the indicated days and (b) comparison between the healed areas of the wounds with Ac2-26 treatment with the healed areas of the wounds with alginate treatment on each specific day.

Standardized digital photographs of the wounds were taken with the same distance between camera and pre-anesthetized mouse, for each animal. The photographs were examined using planimetry for objective evaluation for degree of wound healing (Woodley et al., 2007). The total pixels that cover the unhealed areas were drawn onto the digital photographs using a pattern overlay in ImageJ, (ImageJ software, Wayne Rasband, National Institute of Health, Bethesda, MD, USA). The number of pixels covering an open wound area on a given day was divided by the number of pixels spreading over the initial wound on day 0 to obtain the percentage of closure. Percentage, rather than actual distance (*e.g.*, mm), of wound closure was calculated from the measured wound areas (pixel density). This method allows more accurate measurements of the wounds, considering the fact that certain margin of errors during surgical procedures may exist among different experimental groups or even among the three mice within the same group (Cheng et al., 2012).

Data of the study are presented as mean ± SD. Statistical significance for comparisons was determined by the Student's 2-tailed *t* test. A P value of equal or less than 0.05 was considered statistically significant.

2.6. Study approval

All animal experiments were performed under protocols that followed the Italian and European Community Council for Animal Care (DL. no. 116/92).

3. Results and discussion

3.1. Preparation and characterization of the peptide loaded hydrogels

Preliminary rheological study on polymer solutions were conducted in order to identify a range of polymeric concentration suitable for both easy topical gel administration and reduced removal problems on living tissue. As shown in Table 1, limits for high M alginate and LMW chitosan solutions were fixed at 2.5% and 5.0% (w/w), 3.5% and 6.5% (w/w), respectively. In fact, for these solutions zero shear viscosity (η_0) ranged between 4100 and 9500 Pa s enabling easy administration and retention of Ac2-26 loaded gels into wound cavity. In fact, higher η_0 would not allow clean deposition of gel into the wound bed whereas lower values caused gel leaking from the cavity.

Alginate and chitosan gels were loaded with Ac2-26 peptide in order to obtain solutions at fixed concentration. On the basis of our previous results on the pro-migratory effects of the peptide on

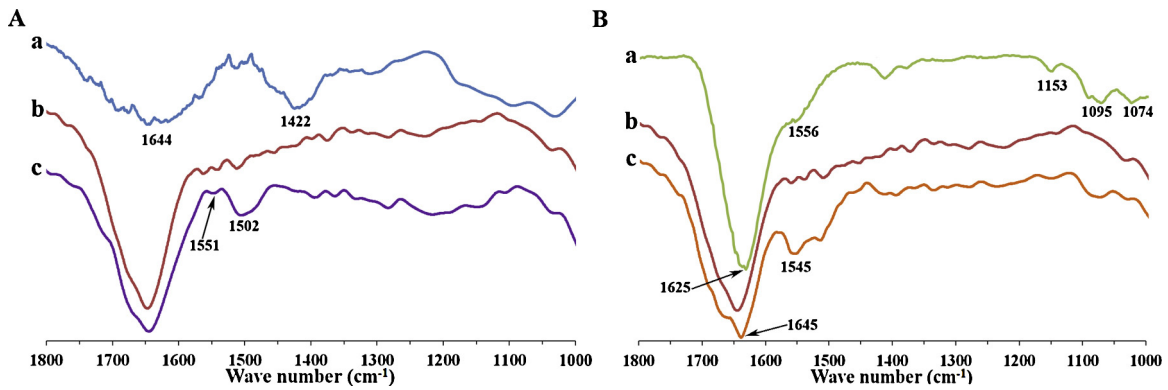


Fig. 1. FTIR spectra of alginate and chitosan Ac2-26 loaded hydrogels in comparison with polymer and pure peptide solutions. Panel (A) 5.0% (w/v) high M content alginate solution (a), 0.5 μM AC2-26 solution (b) and ACA50-500 hydrogel (c). Panel (B) 6.5% (w/v) LMW chitosan solution (a), 0.5 μM AC2-26 solution (b) and ACC65-500 hydrogel (c).

WS1 human fibroblasts in a *in vitro* wound healing assay (Bizzarro et al., 2012b), Ac2-26 gels prepared at three different concentrations (100 nM, 500 nM and 1 μM) were tested in order to assess peptide stability into the polymeric gel matrix over time. Moreover, to evaluate the Ac2-26 tendency to interact with alginate or chitosan, quantitative LC/MS analyses were performed to measure the amount of recovered peptide following 6 h of incubation into different gels. Specifically, two different concentrations of the peptide (100 nM and 1 μM) were loaded into four different gels (2.5% and 5.0% alginate, 3.5% and 6.5% chitosan). For all the alginate hydrogels, regardless of Ac2-26 or alginate concentration, peptide recoveries between 82 and 96% were achieved whereas significant amounts of the peptide were not recovered when Ac2-26 was loaded into chitosan gels (average recovery less than 15%) probably due to a chemical interaction between peptide and chitosan amino groups.

In order to verify the presence of such interaction in the gels, FT-IR studies on the different formulations were conducted. The characteristic peaks of chitosan and alginate are reported in Fig. 1. Alginate carboxyl peaks at 1644 and 1422 cm^{-1} corresponding to symmetric and asymmetric COO stretching vibration were shifted at 1551 and 1502 cm^{-1} in Ac2-26 loaded alginate gel (Fig. 1Aa–c) probably due to an extensive hydrogen bonding in a crowded environment such as the concentrated alginate solution (Piotto, Di Biasi, Concilio, Castiglione, & Cattaneo, 2014) between Ac2-26 and alginate carboxyl group. The FTIR spectrum of chitosan in Fig. 1Ba shows the peaks of amide bond at 1625 cm^{-1}, $-NH_2$ bending of the protonated amino peaks at 1556 cm^{-1} due to the presence of N-deacetylated groups and the absorption bands at 1153, 1095 and 1074 cm^{-1} characteristic of the stretching of its skeletal saccharine structure. N-related peaks were shifted in Ac2-26 loaded LMW chitosan gel, amide peak into singlet band at 1645 cm^{-1} and the amino signal to a broad peak at 1545 cm^{-1}, both not existing into the Ac2-26 peptide spectrum.

Changes in the bands of the amide bonds and amino groups can be attributed to an ionic interaction between the amino group of chitosan and the region from Asn 16 and Glu 20 of Ac2-26 peptide

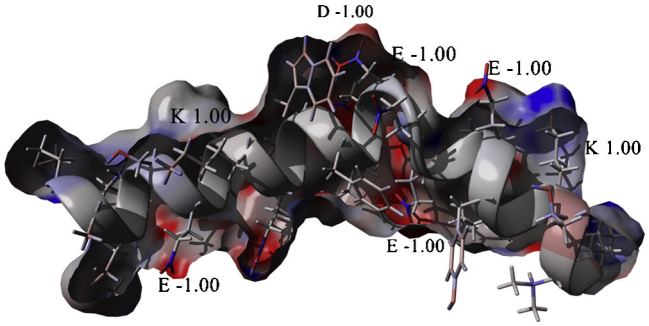


Fig. 2. Ac2-26 solvent accessible surface mapped at chitosan hydrogel pH: red represents negative charge, blue indicates positive charge. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

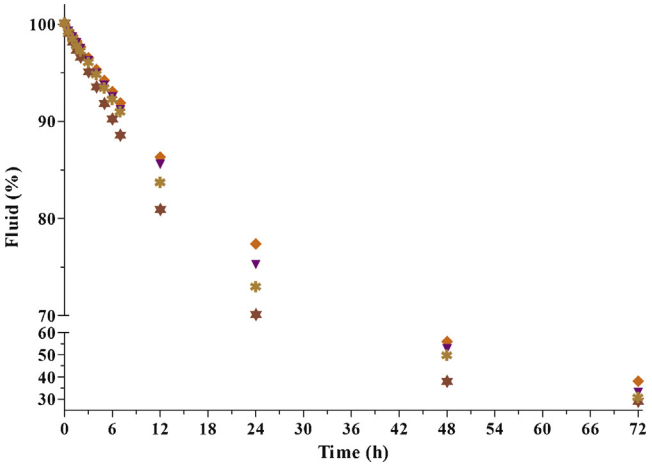


Fig. 3. Water loss of 1 μM AC2-26 loaded hydrogels at different concentrations: high M content alginate 5.0% (-◆-) and 2.5% (-▼-), LMW chitosan 6.5% (-✱-) and 3.5% (-✱-). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

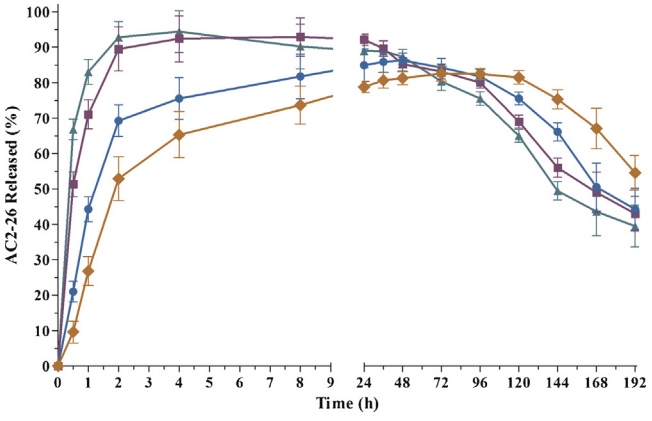
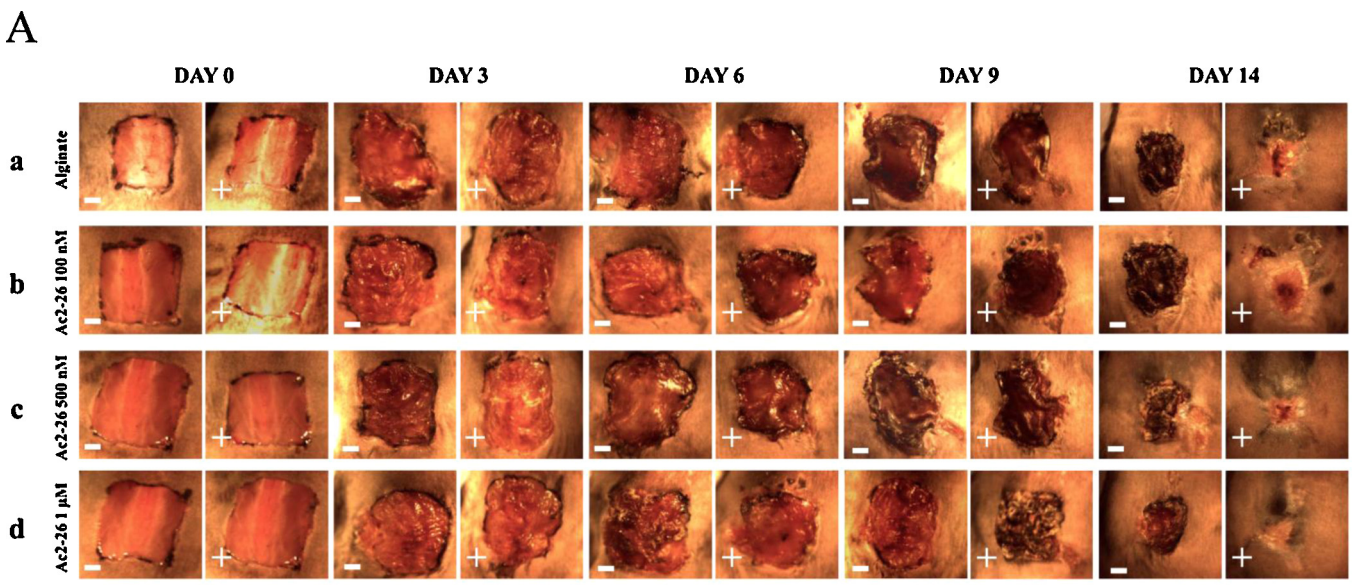


Fig. 4. Release profiles of AC2-26 loaded alginate gels at different concentrations: ACA25-100 (-▲-), ACA25-1000 (-■-), ACA50-100 (-●-) and ACA50-1000 (-◆-). Mean ± SD; (n = 6). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



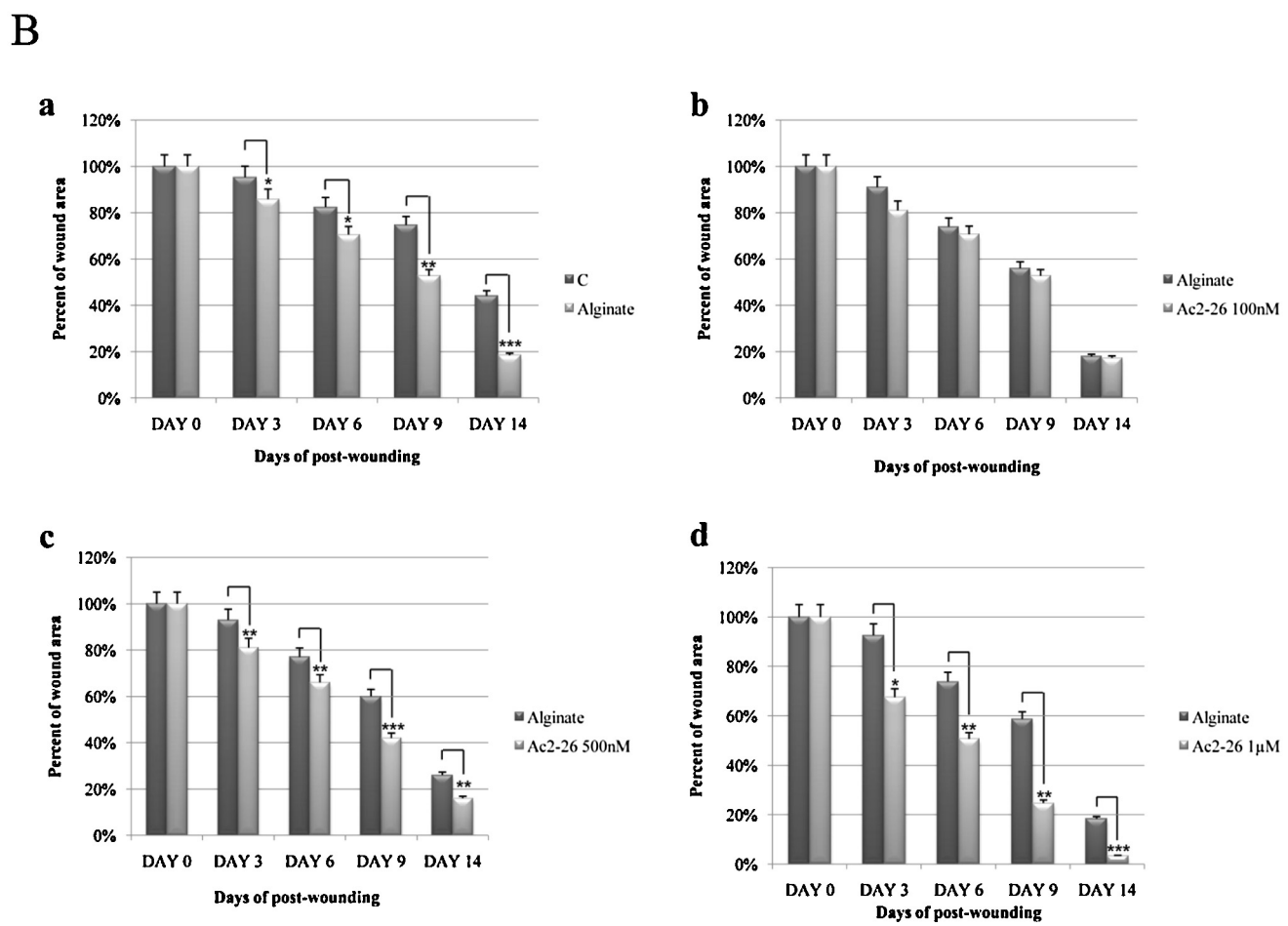


Fig. 5. (A) Ac2-26 loaded alginate hydrogel ameliorates skin wound repair in acute skin wound healing. Full-thickness skin wounds (1 cm × 1 cm) in C57BL/6 mice were treated with: (a) 5.0% blank alginate hydrogel or loaded with an optimized concentration of (b) Ac2-26 100 nM, (c) Ac2-26 500 nM, (d) Ac2-26 1 μM ($n = 3$ mice per peptide, per experiment. Plus signs indicate treated mice, and minus signs indicate control mice. The images of 1 representative experiment are shown. (B) Measurements of blank alginate hydrogel *vs* Ac2-26 loaded alginate hydrogel in acute wound healing. Percentage of the accelerated wound closure at days 0, 3, 6, 9, and 14 after treatments with ACA50s hydrogel formulations or blank alginate hydrogel *vs* control ((a)–(d)) (mean ± SD). * $P \leq 0.05$, compared with control or blank alginate hydrogel.

that in the ACCs gels bear a negative charge of −2 (Scrima et al., 2014), as shown in Fig. 2.

Such negatively charged portion of the peptide could allow an efficient binding onto the positive chitosan chain that might prevent Ac2-26 loaded chitosan gel to release the peptide under mild pH condition agreement with the recovery data obtained by LC/MS. Moreover, the persisting in ACCs formulation of the peak related to the chitosan amino groups at 1153 cm^{-1} suggests the presence of an interaction between chitosan and Ac2-26 in agreement with the molar ratio between them.

In order to mimic wound clinical conditions *in vitro* and the environment in which the different formulations could be administered, a simulated wound fluid (SWF) was used to test fluid equilibrium of Ac2-26 loaded gels over time. Fluid loss was evaluated as the weight decrease of the different formulations when in contact with SWF. As shown in Fig. 3, all formulations exhibited exponential weight loss over 24 h and a linear (slower) fluid loss kinetic between 24 and 72 h. This may explained by the formation of a polymer enriched layer on the surface of the gel able to retard further water desorption (Şen & Güven, 2002). However, mid-value of deswelling (fifty percent of fluid loss) was reached in about 42 or 55 h for chitosan and alginate gels, respectively; whereas, dry state was reached between 120 and 132 h depending on polymer concentration. Moreover, fluid loss was also related to polymer concentration. In fact, the higher the concentration the slower the desorption kinetic.

3.2. Peptide release studies

In order to point out any differences in Ac2-26 release behaviour from different gel formulations drug release profiles were monitored using slide-a-lyzer mini dialysis units, 10 K molecular weight cut off using SWF (simulated wound fluid) in the acceptor chamber. Fig. 4 reports the release curves of alginate AC2-26 loaded gels. As expected, release behaviour was directly correlated to peptide and polymeric concentration. In fact, alginate/peptide formulation ACA25 exhibited the faster permeation rate, achieving maximum release into the acceptor compartment between 4 and 8 h according to the increasing in polymer concentration, whereas ACA50 formulations between 48 to 72 h. ACA25-500 as well as ACA50-500 demonstrated profiles almost superimposable to ACA25-1000 and ACA50-1000 (data not shown). However, total release of the peptide was never achieved even for the less concentrated polymeric gels probably due the interaction of the Ac2-26 *via* hydrogen bonding with alginate carboxyl residues, as demonstrated by FTIR analyses.

All ACA gels exhibited a reduction in peptide concentration starting between 24 and 96 h, according with peptide release rate that might be associated with AC2-26 degradation in SWF at 37 °C. Moreover, alginate/peptide gels exhibited a burst effect within 2 h related to alginate concentration, resulting in the release of about 90% of the peptide for ACA25 gels while after 2 h ACA50-100 and ACA50-1000 formulations released 69 and 52% of the loaded peptide, respectively. Such intensive release of the peptide in the first hours of administration, followed by a prolonged release, maybe very useful at the beginning of the healing process that could benefit of the strong anti-inflammatory and pro-migratory effects related to high concentration of Ac2-26 (Teixeira, Mimura, Araujo, Greco, & Oliani, 2013).

In spite of similar viscosity behaviour, diffusion of AC2-26 from chitosan/peptide formulations was very poor with very slow release rate, reaching around 10% in about 72 h even for the most concentrated peptide gel (data not shown). This phenomenon was correlated to the strong interaction between Asn 16 and Glu 20 of Ac2-26 and LMW chitosan, as highlighted by FTIR analyses (Fig. 1).

3.3. In vivo wound healing test

Impaired cell migration may contribute to the poor wound healing observed in diabetic patients and may represent a target of therapeutic intervention. To date, becaplermin (recombinant human PDGF-BB) is the only US FDA-approved therapy, and it shows modest efficacy (15% of wound closure *vs* control at day 21th post-wounding at 100 μg/g of PDGF-BB), is costly, and has the potential to cause cancer in patients. Previous studies have been demonstrated that the administration of ANXA1 N-terminal derived peptide Ac2-26 could significantly improve the healing of wound in models also in high glucose conditions (Bizzarro et al., 2012b). On the other hand, high M content alginate has been widely applied as dressing material and drug carrier demonstrating an acceleration of the wound healing process (De Cicco et al., 2014b; Thomas, Harding, & Moore, 2000). Therefore, the combination of Ac2-26 peptide and alginate should have synergistic effect in healing process.

Fig. 4 shows the evolution of wound repair process for each group of mice used during the experimental plan that closed with the time evolution. Application of ACA50s hydrogels and unloaded **alginate hydrogels** slightly accelerated the healing on the wounded area after 3 days of treatment as compared with control (saline solution treatment, Fig. 5A). However, the wound reduction showed no significantly difference between unloaded alginate hydrogels and Ac2-26 100 nM/alginate group (Fig. 5Bb). Fourteen days later, the wound areas for ACA50-500 and ACA50-1000 groups were significantly decreased with almost completely wound closure compared with control (from 26% to 16%) and unloaded alginate hydrogel (from 18% to 3%). These therapeutic properties of ANXA1 on wound closure were observed in other tissues and could be due to the well known autocrine and paracrine anti-inflammatory effects of its N-terminus peptide, Ac2-26, that is released from the full length protein through regulated proteolysis (Bizzarro et al., 2012b; Perretti & D'Acquisto, 2009). The biological effects of ANXA1 and its cleavage product Ac2-26 peptide are mediated by formyl peptide receptors (FPRs) that regulate innate inflammatory responses and promote cell motility (Leoni et al., 2013).

Our results clearly suggest that ANXA1 mimetic peptide Ac2-26 in combination with high M alginate may have therapeutic effects and could represent a new bioactive compound with low dose/high efficiency wound healing activity (97% of wound closure *vs* control at day 14th post-injury at 1 μM dose) in the topical drug controlled release formulation. If both such efficacy and duration of the Ac2-26/alginate hydrogels action could translate into humans, it may significantly improve patient life and help to reduce the overall cost of clinical wound treatments. Future studies will be needed to address this point.

4. Conclusions

Hydrogels based on high M content alginate or LMW chitosan incorporating the ANXA1 mimetic peptide Ac2-26 were successfully prepared in view of their use as wound dressing.

Considering viscosity, gel mid-value deswelling and dry state time point (120–132 h) of the hydrogels it is reasonable to conclude that they were suitable dressing to avoid removal problems and living tissue dehydration over time. FTIR studies supported by LC–MS results suggest a binding between the negatively charged portion of the peptide and the positive chitosan chain as an extensive hydrogen bonding between Ac2-26 and alginate that stabilizes the peptide when incorporated into the hydrogels. Such interactions are able to slow down peptide release from alginate and prevent its permeation from chitosan. *In vivo* wound healing tests

on mice clearly show an acceleration of wound closure compared with the topical application of unloaded alginate (especially with 0.5 and 1 μM Ac2-26/alginate formulations) allowing 97% of wound closure at day 14th. It can be concluded that alginate hydrogel is a useful polymeric carrier for ANXA1 mimetic peptide Ac2-26 and the high M alginate/Ac2-26 formulations might have great potential application as dressing in wound healing processes.

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