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Title: Freeze dried chitosan acetate dressings with glycosaminoglycans and traxenamic acid

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Abstract: Bleeding control plays an important role to increase survival in the early phase after a traumatic event.
The aim of the present work was the development of hemostatic sponge-like dressings based on chitosan, in association with glycosaminoglycans (GAG as chondroitin sulfate or hyaluronic acid) and the improvement of their hemostatic performance by loading tranexamic acid (TA). The dressings were prepared by lyophilizazion and were characterized for mechanical, hydration, bioadhesion properties and morphology. Moreover FTIR analysis was performed to understand the interactions bet ween the different polyelectrolytes present in the dressing. Clotting was investigated in vitro by using rat whole blood. Moreover in vitro biocompatibility and proliferation were evaluated towards fibroblasts. Ex vivo proliferation properties were assessed by using human skin.
All the dressings were characterised by mechanical, hydration and bioadhesion properties suitable to be applied on bleeding wounds and to absorb bleeding or wound exudate, avoiding tissue dehydration. TA release was fast and TA and chitosan showed a synergic effect to speed up clotting. The dressings were biocompatible and able to sustain cell proliferation in vitro and ex vivo in human epidermis. In conclusion, sponge-like dressings based on chitosan and GAG and loaded with TA are an effective tool to enhance hemostasis and healing in bleeding wounds.

Cover Letter

Dear Editor,

please consider the revised version of the paper previously entitled "Improvement of hemostatic dressings based on chitosan and glycosaminoglycans by tranexamic acid" and now entitled "Freeze dried chitosan acetate dressings with glycosaminoglycans and traxenamic acid" as suggested. The manuscript was carefully revised considering all the points raised by the reviewers.

The aim of paper submitted is the development of hemostatic sponge-like dressings based on chitosan, in association with glycosaminoglycans (GAG as chondroitin sulfate or hyaluronic acid) and the improvement of their hemostatic performance by loading tranexamic acid (TA).

The significance of the research considers two fundamental aspects: the first one is related to the development of sponge-like dressings with optimal technological properties (mechanical, hydration and bioadhesive), while the second one is related to the possibility to speed up clotting and to enhance wound healing by cell proliferation.

The characterization of these systems involved a multidisciplinary approach considering integrated techniques of pharmaceutical technology, and in vitro/ex vivo models to understand clotting, biocompatibility and proliferation properties (enhancement of in vitro and ex vivo proliferation on fibroblast and human skin biopsy, respectively).

A deeper characterization of the physico-chemical properties of the developed dressings was performed: FT-IR, SEM analysis and acetic acid titration was added in the paper and XRPD analysis was performed but not included into the paper. These evaluations, in line with the requests of the reviewers, allowed to characterize the systems from solid state point of view. These further characterizations completed the comprehension of the behaviour of the developed dressings upon hydration and of the bioadhesion phenomenon.

Thanks in advance for your consideration,

Best regards

Giuseppina Sandri

Highlights

Chitosan and glycosaminoglycan dressings are characterized by mechanical, hydration and bioadhesive properties suitable to be applied on bleeding wounds.

Chitosan and glycosaminoglycan interaction was studied by FTIR and dressing morphology was characterized by SEM.

Chitosan and tranexamic acid have a synergic effect in clot formation.

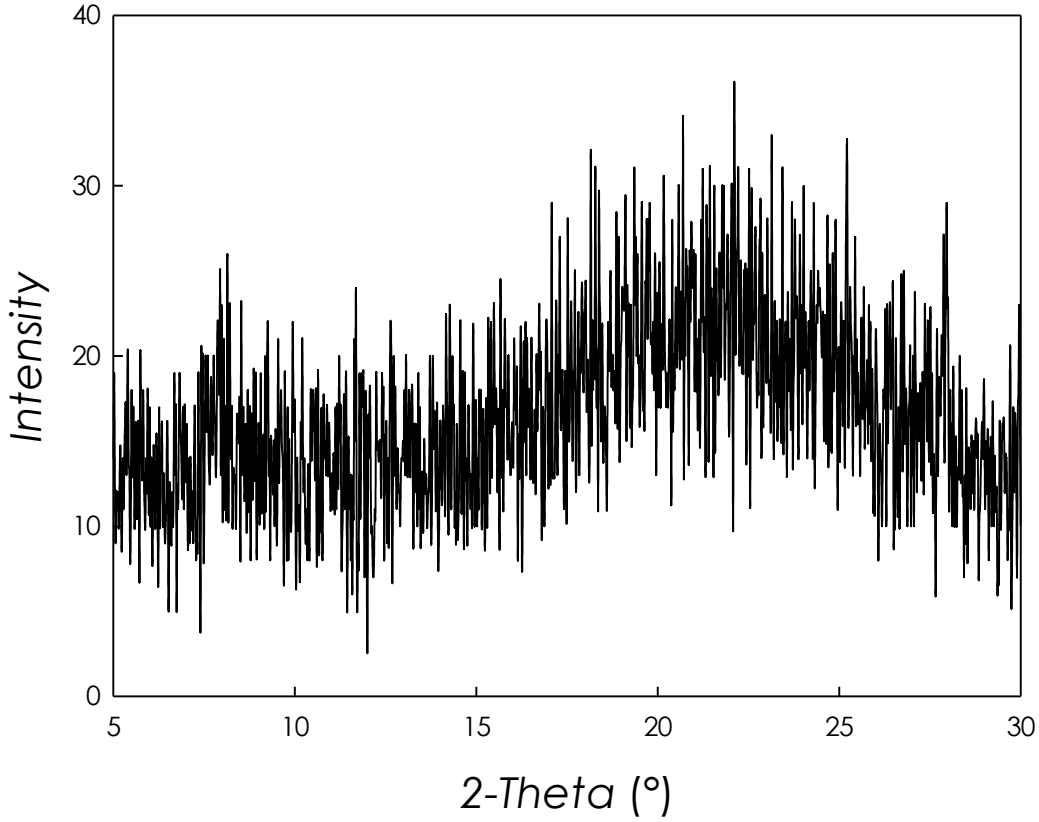
***Response to Reviewers**

The authors wish to thank the reviewers for their fruitful comments to improve the quality of the paper. All the changes in the manuscripts were in red, as requested.

Editor

1. Following the editor's suggestion the title was changed in "Freeze dried chitosan acetate dressings with glycosaminoglycans and traxenamic acid"
2. The reference suggested Bartley et al., 2013 was added and the following comment was added in the text: "Bartley (2013) suggested that the association of chitosan with TA should improve hemostatic control and should lead to improved clinical outcomes in internal surgical wounds."
3. The rationale of the work was better stated in the introduction part and the discussion of the results was improved.

   Acetic acid titration was performed on dressings and the comment of results was added in the text as follows "Moreover lyophilization caused acetic acid evaporation. In particular, acetic acid was added, to form chitosan acetate, in excess: 350 μmoli of acetic acid were added to 110 μmoli of chitosan per each dressing prepared, starting from chitosan at 1% w/w. The titration of acetic acid residues after lyophilization revealed that there was a residues of 5±1 μmoli per each systems independently of the addition of HA or CS. This is conceivably due to the almost total removal of acetic acid in excess (not involved in chitosan salification)."
   Paracrystallinity of the dressings was checked by means of XRPD analysis. XRPD measurements were performed using a D5005 Bruker diffractometer (Karlsruhe, Germany) (CuKα radiation, λ(Kα1) = 1.54056 Å; voltage of 40 kV and current of 40 mA) equipped with a θ-θ vertical goniometer, Ni filter, monochromator and scintillator counter. The patterns were recorded at room temperature in step scan mode (step size: 0.020°, counting time: 3 s per step) in the $5 < 2\theta° < 35$ angular range.
   The patters did not present any peaks to suggest the lacking of paracrillanity. The data have not be included in the text but hereafter as example you can see the CH 1 HA patter.



The bioadhesion paragraph in the results and discussion part was completely rewritten, also following the suggestions of reviewer #1, as follows. Figure 3 reports maximum force of bioadhesion (mN) all the dressings prepared: a) without lyoprotectant, b) with glycerol and c) with mannitol as lyoprotectans.
As for dressings without lyoprotectants (Fig. 3 a), chitosan concentration influenced bioadhesive behavior: CH 2 sponge-like dressing (containing higher chitosan amount) did not show bioadhesive

propensity (Fmax measured without biological substrate was not significantly different with respect to the value in presence of biological substrate – egg shell membrane) while CH 1 dressing (containing chitosan at lower amount) was characterized by good bioadhesive properties. Such a behavior is probably due to a different degree of entanglement between chitosan polymeric chains. In particular, the lowest chitosan concentration the lowest the entanglement and the highest the capability to form a bioadhesive joint with the biological substrate (Rossi, Ferrari, Bonferoni, Caramella, 2001).
Since zeta potential analysis confirmed that there was not a complete neutralization of CH with GAGs and both CH-HA and CH-CS had positive zeta potentials, the presence of GAG in dressings significantly enhanced the bioadhesive properties. The interaction between chitosan and GAG probably assisted bioadhesive joint consolidation, considering a higher concentration of carboxylic and OH residues in these systems with respect to that of CH 1. The highest presence of these groups able to form hydrogen bonds could consolidate bioadhesive properties of chitosan, that are mainly due to its positive charge density. Chitosan and GAG physical mixtures (CH-HApm, and CH-CSpm) did not show bioadhesive properties: these are easily inferred by the negligible solubility properties of chitosan at pH 7.2 phosphate buffer (hydration medium chosen to mimic wound bed exudate). Moreover CS or HA, as powders, were also considered and they showed a lack of bioadhesion in those conditions: this was probably due to slower hydration of HA during the test with respect to the lyophilized dressings and to the poor bioadhesive strenght of CS mainly caused by its low molecular weight. In presence of lyoprotectans, both mannitol and glycerol (Fig. 3 b and c, respectively), the bioadhesive properties were less pronounced and were significant only for HA and CS dressings with mannitol. The presence of lyoprotectans, especially of mannitol, that increased system stiffness and probably decreased polymer chain mobility, could impair polymer capability to interact with the biological substrate.
Also bioadhesion is a crucial characteristic that should favor an intimate and prolonged contact between wound dressings and lesion, favoring blood absorption and avoiding formulation detachment to increase the hemostatic potential.
Moreover zeta potential evaluation and SEM analysis to study system morphology were added.

Reviewer #1

L111/Table 1: According to reviewer's suggestions, average molecular mass of both CS and HA was included in the materials section as follows: "The following materials were used: Chitosan (CH) low MW 251000 Da, deacetylation degree 98%, maximum charge density: number of positively charged functional group per repeated unit: 0.98 (ChitoClear, Siiiglufjordur-Iceland); Hyaluronic Acid (HA) low MW 212000 Da; maximum charge density: number of negatively charged functional group per repeated unit: 0.5 (Bioiberica, Barenz, Italy); Chondroitin sodium sulfate bovine 100 EP (CS) low MW 14000 Da, mixture of A (chondroitin 4 sulfate) and C (chondroitin 6 sulfate); maximum charge density: number of negatively charged functional group per repeated unit: 1 (Bioiberica, Barenz, Italy); Trans-4 (amino-methyl) cyclohexanecarboxyilic acid (Sigma Aldrich, Milan-Italy); Glycerol (g) 30° Be (Carlo Erba, Italy); D-Mannitol (Fluka, France)"
The charge density (number of negatively charged functional grouped per repeated unit) and the charge molar ratio between the two polyelectrolytes were also added for both CS and HA in the results and discussion section. "charge density (positive) is 0.006 mole/g for chitosan, 0.0013 mole/g for HA (negative) and 0.002 mole/g for CS (negative) with a clear excess of positive charges, in both cases. The charge molar ratio of CH – HA was 4.6 while that of CH – CS was 3."

The rationale about the employment of acetate rather than hydrochloride was assessed in the experimental part 2.2.1. Sponge-like dressing preparation as follows: . Acetate was used as counterion to obtain chitosan salification in mild pH conditions and it was preferred to hydrochloric acid because chitosan fragmentation could occur in HCl environment also at low molarity (Sabnis, Block, 2000).

L117/L118: According to reviewer's suggestions, in the experimental part the paragraph "2.2.1. Sponge-like dressing preparation" was rewritten including acetic acid concentration details and the visual observation of the systems prepared as follows "CH was hydrated in acetic acid 1% w/w (glacial acetic acid, Sigma Aldrich, Italy) under gentle stirring at room temperature. Acetate was used as counterion to obtain chitosan salification in mild pH conditions and it was preferred to hydrochloric acid because chitosan fragmentation could occur in HCl environment also at low molarity (Sabnis, Block, 2000).
HA and CS were hydrated in distilled water under gentle stirring at room temperature. The two polymeric solutions were mixed 1:1 weight ratio to obtain polymeric mixtures. Systems containing CH alone were mixed

1:1 weight ratio with bidistilled water. Final acetic acid concentration in the polymeric systems was 0.5% w/w. CH 1 - HA and CH 1 - CS mixtures and CH 1, were subjected to zeta potential evaluation. Measurements were carried out at 25°C by means of a Malvern Zetasizer Nano ZS90 (Malvern Ltd., UK). HA and CS solutions, prepared in the same concentrations used for CH 1 - HA and CH 1 - CS mixtures but without CH were also tested.
In two series of preparations of CH 1 - HA and CH 1 - CS mixtures and CH 1, glycerol or mannitol were added as lyoprotectants. Polymeric solutions appeared transparent without visible precipitate. As for TA loaded systems drug was added to the polymeric mixture at 0.75% w/w. Two ml of each mixture were poured into each well of 12-well plate (well area 3.8 cm2). In the TA loaded systems the drug was 4 mg/cm2. All the polymeric solutions were frozen at -40°C overnight and freeze-dried (Heto 15, Analitica De Mori, I) for 24 h. All the dressings prepared were 5 mm thick (sd ± 0.52).
CH, HA and CS were at 5 mg/cm2 concentration in CH 1 dressings; CH was at 10 mg/cm2 concentration in CH 2 dressings, glycerol was at 1.3 mg/cm2 while mannitol was at 10.5 mg/cm2 .
Dressings were re-solubilized in distilled water to check the eventual residues of acetic acid (2 g of distilled water per each dressing). pH of each sample was measured by means of a pHmeter (pH 210 Microprocessor, Hanna Instruments, Italy) and the acetic acid excess was titrated by means of acid-base titration by using phenolphthatelin as indicator and NaOH 0.1 M (Carlo Erba.Italy).

L214: in the experimental part paragraph “2.2.2.8. In vitro biocompatibility and proliferation” requested details about sample preparation were added as follows “ After 24 h cells were washed with saline solution and placed in contact with 200 µl of CH, CH-CS and CH-HA polymeric mixtures (prepared as reported in the section 2.2.1.) at 1:20 (CH, CS or HA at 500 µg/ml, solubilized in cell growth medium) and 1:50 (CH, CS or HA at 200 µg/ml, solubilized in cell growth medium) dilutions with growth medium (GM).

L263-268: According to reviewer’s suggestions, the section was completely rewritten: a discussion about the PECs formation was added including also references about the topic and the concept of guest-host was discussed, as follows: “The increase of chitosan amounts in the dressings increased the mechanical resistance of the formulations: stiffness was significantly greater when lyoprotectants (glycerol or mannitol) were included in the formulations. The presence of HA in the dressings caused a significantly increase in the mechanical resistance and HA based formulation with mannitol, as lyoprotectant, was characterized by the higher Fmax value to indicate a more rigid structure. This behavior could probably due to the high molecular weight of HA, with respect to CS, that could dramatically strengthen the tridimensional network of the system. It is conceivable that HA interacted with chitosan during the preparation procedure, forming a polyelectrolyte complex (PEC) between chitosan and hyaluronate. Since both CH and HA have similar molecular weight the interaction between the two polymers should be able to form a highly crosslinked structure.
On the contrary, the presence of CS in the formulation did not significantly change the stiffness of the dressings. This is probably due to the resulting PEC formed by an interaction between CS and CH: CS is characterized by a molecular weight 18 folds lower than that of CH, moreover sulfate groups of CS (more acid with respect to carboxylic group) could cause a coiled structure less prone to polymer chain entanglements. This probably rendered the dressing structure less stiff.
As reported in literature, structure and stability of PECs strongly depend on different parameters such as concentrations of each polyelectrolyte, charge molar ratio, charge density, molar masses and chain flexibility but depend also on extrinsic parameters as pH, ionic strength and/or temperature (Kabanov, 2005; Feng, Leduc, and Pelton, 2008; Le Cerf, D., Pepin, A. S., Niang, P.M., Cristea et al., 2014).
According to all these parameters, mixing oppositely charged polyelectrolytes could give two types of PECs dependently of charge stoichiometry: soluble PECs, leading to stable and transparent solutions, and insoluble PECs, with or without precipitation. The soluble PECs are obtained with an excess of anionic or cationic charges (Feng, Leduc, and Pelton, 2008; Kabanov and Zezin, 1984; Schatz, Domard, Viton, Pichot, et al., 2004) and this was the case of CH-HA and CH-CS. In fact, charge density (positive) is 0.006 mole/g for chitosan, 0.0013 mole/g for HA (negative) and 0.002 mole/g for CS (negative) with a clear excess of positive charges, in both cases. The charge molar ratio of CH – HA was 4.6 while that of CH – CS was 3.
Moreover Denunziere, Ferrier, and Domard (1996) reported interaction between CS and CH was independent of CS sulfate substitution on 4 or 6 carbons.

Zeta potential evaluation was also added in the text (experimental part and Results and discussion part) as follows: This was confirmed by zeta potential evaluation. CH 1 had a positive zeta potential of 53.2±1.9 (mean value±sd; n=3) while HA and CS as solutions showed negative zeta potentials of -27.9±0.9 and -16.8±1.2, respectively (mean value±sd; n=3). CH 1 – HA and CH 1 – CS were characterized by positive zeta potentials of 39.3±0.3 and 25.3±0.8, respectively (mean value±sd; n=3). These results supported that the

interaction between CH and GAGs did not cause a neutralization of the two polyelectrolytes mixed (CH and HA or CS) and an excess of positive charges was present.

Acetic acid titration was performed and the results were commented as follows: “Moreover lyophilization caused acetic acid evaporation. In particular, acetic acid was added, to form chitosan acetate, in excess: 350 μmoli of acetic acid were added to 110 μmoli of chitosan per each dressing prepared, starting from chitosan at 1% w/w. The titration of acetic acid residues after lyophilization revealed that there was a residues of 5±1 μmoli per each systems independently of the addition of HA or CS. This is conceivably due to the almost total removal of acetic acid in excess (not involved in chitosan salification).2

Morphology was also assessed by means of SEM and the results were commented as follows: To understand if the system tridimensional networks could affect hydration and bioadhesion properties, morphology was assessed.
Figure 4 reports SEM images of transversal sections of CH 1, CH 1- HA and CH 1 – CS unloaded dressings and TA loaded dressings without lyoprotectans, and CH 1, CH 1- HA and CH 1 – CS dressings with glycerol and with mannitol.
CH 1 dressing showed a beehive structure with polyhedric cavities interconnected by pores having oval or round shapes. The cavities had dimensions of about 500 μm while the pores ranged from 50 to 200 μm. The presence of GAG did not change the system morphology while it markedly decreased the cavity dimensions that were around 200-300 μm and furthermore the pore diameters that were of about 50 μm. This difference in system porosity conceivably contributed to their hydration behavior. The TA loading did not modify the structure of the dressings.
The presence of glycerol as lyoprotectant did not substantially change dressing structure while the presence of mannitol caused a partially loss of polyhedric cavities and the structure resembled much more randomly organized sheets without marked modifications of the porosity. This modification could be responsible to the system behavior upon hydration and bioadhesion.
Since lyoprotectans did not markedly improve the technological properties of the dressings and considering mechanical, hydration and bioadhesive properties, lyoprotectant free systems prepared using chitosan at 1% w/w were considered for the further characterizations.

L289-301: the protonation of CH after lyophilization was assessed in the dressings by means of FTIR analysis and also to study interpolymer interactions. The results of FTIR analysis were commented as follows:
Figure 5 reports the FT-IR spectra of CH 1, CH 1 – HA and CH 1 – CS HA and CH+CS dressings and of CH powder (in the inset) as comparison.

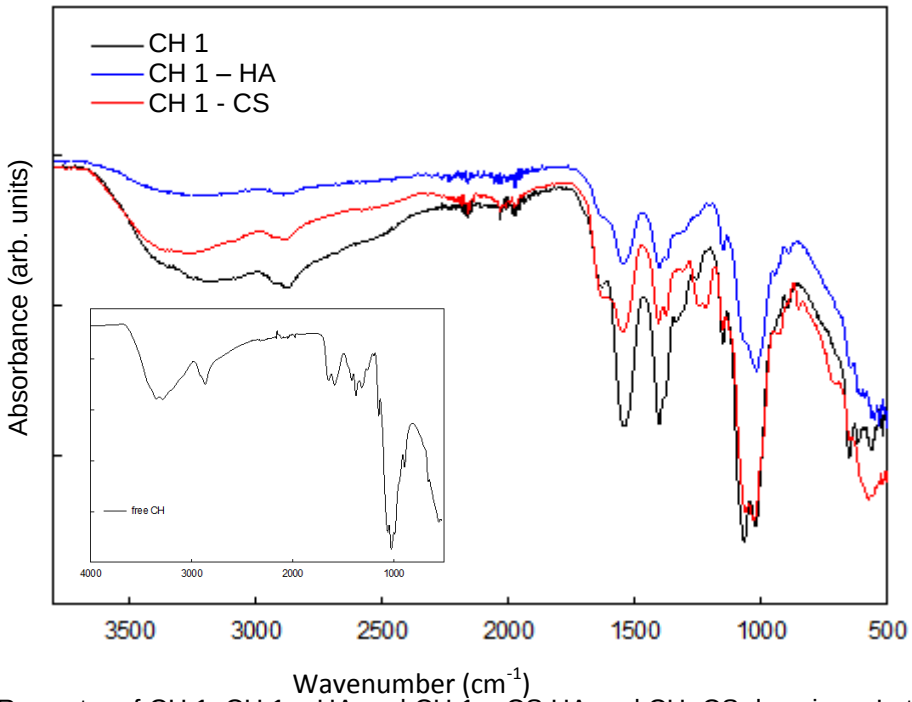


Figure 5: FT-IR spectra of CH 1, CH 1 – HA and CH 1 – CS HA and CH+CS dressings. In the inset the FTIR spectrum of CH powder (free CH) is reported as comparison.

The three spectra shared many common features both in the number and position of the peaks and seemed to be dominated by the FT-IR pattern of CH (free CH as powder), where the region around 1630 and 1540 cm^{-1} is typical of the amide I and amide II stretching and bending modes (Radhakumary, Antony, Sreenivasan, 2011; Jayakumar, Prabhakaran, Nair, Tamura, 2010). Interestingly, the position of these peaks shifted to lower frequency with respect to free CH (i.e. not interacting with acetate ions) shown Figure 1 inset. In particular, the –C=O stretching of the amide I in free CH moved from 1652 to 1634 cm^{-1} when it interacted with acetate ions and it remained in the same position even in the CH 1 - HA and CH 1 - CS systems. Moreover, a new peak around 1404 cm^{-1} (correlated to the –C-H- bending) appeared in the spectra of CH 1 dressing with respect to free CH and was found also in the spectra of CH 1 - HA and CH 1 - CS. The two mentioned spectral features have been correlated to charge-charge neutralization (salification) which most probably occurred between CH and acetate ions and dominated the spectra of all the three systems shown in Figure 1 (Shanti Krishna, Radhakumary, Sreenivasan, 2015).
This suggests that, in presence of acetate ions, the charge-charge interaction, involving chitosan amino groups, was preferentially between CH and acetate and therefore the further addition of HA and CS mostly interact with chitosan through less strong bonding such as polar interactions. Such results are in agreement with zeta potential values determined.

Also Zeta potential and acetic acid titration allowed to understand the protonation of chitosan after lyophilization and the interactions between CH and GAG to interpret the hydration results. The rational of hydration properties at pH 7.2 was better evidenced also in the results part (to mimic wound exudates).

Moreover the impact of cryoprotectant on dressings was added as follows:
Glycerol did not markedly change the hydration properties of CH and CH in association with GAG. On the contrary, mannitol substantially decreased the hydration capacity of the systems: this could be related to stiff structure of the dressings, which could slow down and impair liquid penetration into the dressings.

Figure 3: in the experimental part “blank” was explained as follows: Blank measurements were performed by using a filter paper disc wetted with 100 µl of isotonic saline solution (NaCl 0.9% w/v) instead of biological substrate: this allowed to evaluate cohesive properties of the sample.

L350: the bioadhesion section was rewritten and also the experimental part was experiment was better evidenced that biological substrate egg-shell membrane used to mimic wound bed.

L360-361: the suggested samples as comparison were prepared and tested and the Result and discussion section was updated :
Figure 3 reports maximum force of bioadhesion (mN) all the dressings prepared: a) without lyoprotectant, b) with glycerol and c) with mannitol as lyoprotectans.
As for dressings without lyoprotectants (Fig. 3 a), chitosan concentration influenced bioadhesive behavior: CH 2 sponge-like dressing (containing higher chitosan amount) did not show bioadhesive propensity (Fmax measured without biological substrate was not significantly different with respect to the value in presence of biological substrate – egg shell membrane) while CH 1 dressing (containing chitosan at lower amount) was characterized by good bioadhesive properties. Such a behavior is probably due to a different degree of entanglement between chitosan polymeric chains. In particular, the lowest chitosan concentration the lowest the entanglement and the highest the capability to form a bioadhesive joint with the biological substrate (Rossi, Ferrari, Bonferoni, Caramella, 2001).
Since zeta potential analysis confirmed that there was not a complete neutralization of CH with GAGs and both CH-HA and CH-CS had positive zeta potentials, the presence of GAG in dressings significantly enhanced the bioadhesive properties. The interaction between chitosan and GAG probably assisted bioadhesive joint consolidation, considering a higher concentration of carboxylic and OH residues in these systems with respect to that of CH 1. The highest presence of these groups able to form hydrogen bonds could consolidate bioadhesive properties of chitosan, that are mainly due to its positive charge density. Chitosan and GAG physical mixtures (CH-HApm, and CH-CSpm) did not show bioadhesive properties: these are easily inferred by the negligible solubility properties of chitosan at pH 7.2 phosphate buffer (hydration medium chosen to mimic wound bed exudate). Moreover CS or HA, as powders, were also considered and they showed a lack of bioadhesion in those conditions: this was probably due to slower hydration of HA during the test with respect to the lyophilized dressings and to the poor bioadhesive strenght of CS mainly caused by its low molecular weight. In presence of lyoprotectans, both mannitol and glycerol (Fig. 3 b and c, respectively), the bioadhesive properties were less pronounced and were significant only for HA and CS dressings with mannitol. The presence of lyoprotectans, especially of mannitol, that increased system

stiffness and probably decreased polymer chain mobility, could impair polymer capability to interact with the biological substrate.
Also bioadhesion is a crucial characteristic that should favor an intimate and prolonged contact between wound dressings and lesion, favoring blood absorption and avoiding formulation detachment to increase the hemostatic potential.

L445: according to reviewer's comment procoagulant activity of CS and HA were considered in the paragraph and references were added to support these findings.
The paragraph was rewritten as follows:
Figure 7 shows % hemoglobin absorbance profiles as function of time evaluated for a) unloaded dressings and b) TA loaded formulations.
A higher degree of hemoglobin absorbance indicates a slower clot formation rate.
Whole blood clotting formation was relatively slow and the hemoglobin absorbance reached the minimum level after about 420 s. A faster clot formation occurred in presence of all the dressings and 30 s immediately after the contact between blood and formulations the % of free hemoglobin was around 20-50%. The minimum level of hemoglobin absorbance was reached after about 300 s. Chitosan capability to bind and aggregate platelets causing agglutination of erythrocytes and activation of hemostasis was predominant in all the formulation considered also in presence of GAG.
TA is a synthetic analogue of lysine and its antifibrinolytic activity is related to reversible bindings with four to five lysine receptor sites on plasminogen or plasmin: this prevents plasmin from binding to and degrading fibrin and preserves clot structure. TA showed a synergic activity with chitosan in enhancing clot formation: all the formulations reached the minimum in hemoglobin absorbance in about 180 s, presenting a better clotting performance with respect to unloaded dressings. TA CH 1 dressing allowed to maintain a % of hemoglobin at 420 s not significantly different from initial value, while CH 1 dressing determined a significant increase in % of hemoglobin comparing the initial value with respect to the final one (at 420 s). TA CH1 –HA and TA CH1- CS dressings proved to maintain clot integrity stable from the beginning up to 420 s, while the same compositions unloaded showed a partial clot modification at 90 s with a sharp increase in % of hemoglobin.
The presence of GAG slightly decreased the procoagulant activity of the dressings. In fact, HA and CS are reported in literature as anticoagulant materials. Fragments of HA having a molecular weight lower that 500 kDa, as it is the case, cause clot structure susceptible to mechanical deformations and softening (Komorowicz, Balazs, Varga, Szabo, et al., 2016). CS is a sulfated glycosaminoglycan with heparin-like activity (Pandolfi, M., Hedner, U., 1984). On this basis, the procogulant acitivity of the systems seems exclusively due to chitosan and its synergic effect with TA.

L520: according to reviewer's comment the experimental procedure of in vitro biocompatibility and proliferation was better explained as follows:
"After 24 h cells were washed with saline solution and placed in contact with 200 µl of CH, CH-CS and CH-HA polymeric mixtures (prepared as reported in the section 2.2.1.) at 1:20 (CH, CS or HA at 500 µg/ml, solubilized in cell growth medium) and 1:50 dilution with growth medium (GM) (CH, CS or HA at 200 µg/ml). After dilution, all the samples were transparent without visible precipitate."
Moreover in the paragraph "2.2.1. Sponge-like dressing preparation the details about the apparence of the samples was added as follows: "Polymeric solutions appeared transparent without visible precipitate."

The section was rewritten as follows:
Figure 8 reports % biocompatibility (a) and % of proliferation (b) of all unloaded or TA loaded dressings (polymeric mixtures used to prepare dressings) towards fibroblasts (NHDF) for 3 and 24 h of contact time, respectively, and the amounts of proliferating nuclei/area (c) (positively stained for BrdU) counted for all the unloaded and TA loaded dressings.
Independently of the concentrations considered, all the dressings were characterized by good biocompatibility properties towards fibroblasts also in presence of TA and the cell availability was not significantly different from those obtained with growth medium (GM, standard growth conditions).
Similarly, after 24 h the lower dressing concentrations determined the higher proliferation properties that becomes more evident in presence of tranexamic acid.
These results put in evidence that the presence of the hemostatic drug, TA, did not interfere with cell growth in the experimental conditions considered. Similar results were observed by Cholewinski et al. (2009): carotid artery derived cells maintained their morphology with toxic reaction in presence of TA.
Ex vivo skin cell proliferation (GM) was maximum in the first 24 h and decreased after 72 h and 7 days, except for CS based system that had constant activity (Figure 8 (c)). Unloaded dressings were able to sustain cell proliferation up to 7 days demonstrating a capability to enhance cell proliferation after tissue damage. TA loaded dressings were characterized by the same proliferating properties as unloaded one,

considering CH 1 and CH1 - HA dressings. As for CH1 - CS dressing the presence of TA significantly increased the system proliferation properties after 24 h and 7 days and allowed to obtain the better performance, with a prolonged effect up to one week. This probably was caused by the synergic effect of TA and CS on cell growth: CS was reported as able to enhance fibroblasts and endothelial cells proliferation (Sandri, Bonferoni, Rossi, Ferrari et al., 2015) while TA could stabilize fibrin clot without impairment of cell growth and proliferation (Cholewinski, Dietrich, Flanagan, Schmitz-Rode et al., 2009). At this purpose fibrin clot was conceivably formed just after skin biopsies and it could retain growth factors released by platelets during normal physiological process, on this perspective TA, as fibrinolytic drug, could preserve the clot integrity assisting cell proliferation (Wolberg and Campbell, 2008).

Figure 7: According to the reviewer's comment the figure was deleted

L607-608: the conclusions paragraph was rewritten as follows:
Sponge-like dressings based on chitosan and chondroitin sulfate or hyaluronic acid were prepared by lyophilization and loaded with tranexamic acid. The presence of GAG conceivably caused the occurrence of either chitosan and hyaluronic acid or chitosan and chondroitin sulfate interactions. The presence of glycosaminoglycans decreased hydration properties of the systems in a buffer simulating wound exudate – pH 7.2) and this was primarily due to dressing morphology. Chitosan based dressings had a beehive structure with polyhedric cavities of about 500 μm, interconnected by pores having oval or round shapes (50 to 200 μm) while dressings based on chitosan in association with GAG markedly decreased the cavity dimensions (200-300 μm) and with smaller pore diameters (about 50) μm. Liquid absorption is a key point in dressings to ensure hemostasis, to control wound bed hydration enhancing grunulation phase and healing.
The presence of GAG in dressings significantly enhanced dressing bioadhesive properties considering as biological substrate egg-shell membrane to mimic wound bed. The association of chitosan with GAG allowed the consolidation of bioadhesive joint probably via hydrogen bonds that strengthen chitosan bioadhesion behavior, mainly due to charge-charge interaction between chitosan and the biological substrate. This is a functional property since bioadhesion is fundamental to favor an intimate and prolonged contact between wound dressings and lesion, avoiding formulation detachment and increasing hemostatic potential.
TA release was fast to allow procoagulant function as quickly as possible and moreover to sustain hemostatic activity for prolonged time, to stabilize clot, moreover TA presented a synergic effect with chitosan to speed up clotting formation.
In vitro and ex vivo evaluations on fibroblasts and human skin, respectively, evidenced that the developed dressings enhanced cell proliferation.
Bartley (2013) suggested that the association of chitosan with TA should improve hemostatic control and should lead to improved clinical outcomes in internal surgical wounds. In this paper, sponge-like dressings based on chitosan and GAG and loaded with TA demonstrated to combine both hemostasis and proliferation properties and it seemed promising in control bleeding and healing in wounds as well as in abdominal surgery.

Reviewer #2

1) Hydration properties paragraph was completely reorganized as follows:
   Figure 2 reports buffer (pH 7.2 phosphate buffer, to mimic wound exudates) taken up a function of time for all the dressings prepared: a) without lyoprotectant, b) with glycerol and c) with mannitol as lyoprotectans.
   Considering all the compositions with and without lyoprotectants, chitosan based dressings were characterized by higher buffer taken up vs time profile with respect to those of chitosan and GAG systems; moreover chitosan dressings based on higher CH amounts, showed higher hydration profile even not significantly different from hydration capability of dressing based on lower CH amounts. Dressings based on chitosan and GAG were characterized by significantly lower hydration profiles, independently of both the presence and type of lyoprotectants. All the systems showed a high hydration capacity in the first hour: it is conceivable that chitosan was likely to create a lower pH environment at the dressing/liquid interface and this caused a high liquid absorption probably due chitosan gelation. Subsequently, chitosan poor solubility at neutral pH (pKa 6.5) prevailed over its buffering properties and liquid absorption stopped. The presence of GAG further decreased system hydration probably due to the interpolymer interaction between chitosan and hyaluronic acid or chondroitin sulfate.
   Glycerol did not markedly change the hydration properties of CH and CH in association with GAG. On the contrary, mannitol substantially decreased the hydration capacity of the systems: this could

be related to stiff structure of the dressings, which could slow down and impair liquid penetration into the dressings.
Hydration behavior is a crucial point in dressings to ensure hemostasis, to absorb wound exudate and to avoid wound bed dehydration, enhancing granulation phase and healing.

Moreover SEM analysis were performed and hydration was correlated also with system structure, as follows "To understand if the system tridimensional networks could affect hydration and bioadhesion properties, morphology was assessed.
Figure 4 reports SEM images of transversal sections of CH 1, CH 1- HA and CH 1 – CS unloaded dressings and TA loaded dressings without lyoprotectans, and CH 1, CH 1- HA and CH 1 – CS dressings with glycerol and with mannitol.
CH 1 dressing showed a beehive structure with polyhedric cavities interconnected by pores having oval or round shapes. The cavities had dimensions of about 500 μm while the pores ranged from 50 to 200 μm. The presence of GAG did not change the system morphology while it markedly decreased the cavity dimensions that were around 200-300 μm and furthermore the pore diameters that were of about 50 μm. This difference in system porosity conceivably contributed to their hydration behavior. The TA loading did not modify the structure of the dressings.
The presence of glycerol as lyoprotectant did not substantially change dressing structure while the presence of mannitol caused a partially loss of polyhedric cavities and the structure resembled much more randomly organized sheets without marked modifications of the porosity. This modification could be responsible to the system behavior upon hydration and bioadhesion.
Since lyoprotectans did not markedly improve the technological properties of the dressings and considering mechanical, hydration and bioadhesive properties, lyoprotectant free systems prepared using chitosan at 1% w/w were considered for the further characterizations."

2) Bioadhesive properties paragraph was improved adding comments as follows:
"Figure 3 reports maximum force of bioadhesion (mN) all the dressings prepared: a) without lyoprotectant, b) with glycerol and c) with mannitol as lyoprotectans.
As for dressings without lyoprotectants (Fig. 3 a), chitosan concentration influenced bioadhesive behavior: CH 2 sponge-like dressing (containing higher chitosan amount) did not show bioadhesive propensity (Fmax measured without biological substrate was not significantly different with respect to the value in presence of biological substrate – egg shell membrane) while CH 1 dressing (containing chitosan at lower amount) was characterized by good bioadhesive properties. Such a behavior is probably due to a different degree of entanglement between chitosan polymeric chains. In particular, the lowest chitosan concentration the lowest the entanglement and the highest the capability to form a bioadhesive joint with the biological substrate (Rossi, Ferrari, Bonferoni, Caramella, 2001).
Since zeta potential analysis confirmed that there was not a complete neutralization of CH with GAGs and both CH-HA and CH-CS had positive zeta potentials, the presence of GAG in dressings significantly enhanced the bioadhesive properties. The interaction between chitosan and GAG probably assisted bioadhesive joint consolidation, considering a higher concentration of carboxylic and OH residues in these systems with respect to that of CH 1. The highest presence of these groups able to form hydrogen bonds could consolidate bioadhesive properties of chitosan, that are mainly due to its positive charge density. Chitosan and GAG physical mixtures (CH-HApm, and CH-CSpm) did not show bioadhesive properties: these are easily inferred by the negligible solubility properties of chitosan at pH 7.2 phosphate buffer (hydration medium chosen to mimic wound bed exudate). Moreover CS or HA, as powders, were also considered and they showed a lack of bioadhesion in those conditions: this was probably due to slower hydration of HA during the test with respect to the lyophilized dressings and to the poor bioadhesive strenght of CS mainly caused by its low molecular weight.
In presence of lyoprotectans, both mannitol and glycerol (Fig. 3 b and c, respectively), the bioadhesive properties were less pronounced and were significant only for HA and CS dressings with mannitol. The presence of lyoprotectans, especially of mannitol, that increased system stiffness and probably decreased polymer chain mobility, could impair polymer capability to interact with the biological substrate.
Also bioadhesion is a crucial characteristic that should favor an intimate and prolonged contact between wound dressings and lesion, favoring blood absorption and avoiding formulation detachment to increase the hemostatic potential."

Reviewer #3

1) SEM analysis have been performed
   The results were commented as follows: “To understand if the system tridimensional networks could affect hydration and bioadhesion properties, morphology was assessed.
   Figure 4 reports SEM images of transversal sections of CH 1, CH 1- HA and CH 1 – CS unloaded dressings and TA loaded dressings without lyoprotectans, and CH 1, CH 1- HA and CH 1 – CS dressings with glycerol and with mannitol.
   CH 1 dressing showed a beehive structure with polyhedric cavities interconnected by pores having oval or round shapes. The cavities had dimensions of about 500 μm while the pores ranged from 50 to 200 μm. The presence of GAG did not change the system morphology while it markedly decreased the cavity dimensions that were around 200-300 μm and furthermore the pore diameters that were of about 50 μm. This difference in system porosity conceivably contributed to their hydration behavior. The TA loading did not modify the structure of the dressings.
   The presence of glycerol as lyoprotectant did not substantially change dressing structure while the presence of mannitol caused a partially loss of polyhedric cavities and the structure resembled much more randomly organized sheets without marked modifications of the porosity. This modification could be responsible to the system behavior upon hydration and bioadhesion.
   Since lyoprotectans did not markedly improve the technological properties of the dressings and considering mechanical, hydration and bioadhesive properties, lyoprotectant free systems prepared using chitosan at 1% w/w were considered for the further characterizations.”
2) The characteristics of GAG were added in the text as follows:
   Hyaluronic Acid (HA) low MW 212000 Da; maximum charge density: number of negatively charged functional groups per repeated unit: 0.5 (Bioiberica, Barenz, Italy);
   Chondroitin sodium sulfate bovine 100 EP (CS) low MW 14000 Da, mixture of A (chondroitin 4 sulfate) and C (chondroitin 6 sulfate); maximum charge density: number of negatively charged functional groups per repeated unit: 1 (Bioiberica, Barenz, Italy).
3) The rationale of the paper was better stated in the introduction evidencing the different approach employed in comparison to previously published paper:
   The polysaccharides based systems should be more biocompatible rather than protein ones. In particular Collagen, one of the major components of ECM seems the best candidate to obtain dressings/scaffolds for wound reparation but in literature it is reported as immunogenic material due to possible helical- recognition by antibodies, due to 3D intact triple helix conformation, peculiar aminoacid sequence able to start antibody recognition, presence of non-helical terminal regions (telopeptides) (Lynn, Yannas and Bonfield, 2004; Olsen, Yang, Bodo, Chang et al., 2003). Moreover it could have concerns of species-to-species transmissible diseases (xenozoonoses) (Cataldo, Ursini, Lilla and Angelini, 2008). …….
   Sponge-like dressings should have advantages in application with respect to powdery hemostatic agents, especially in the treatment of intra-abdominal solid organ bleedings or injuries: if hemostatic agents are based on granules (microparticles) can leave residue in the lumen of the vessel and may occlude distal arterial flow. Moreover intraluminal dissemination of the clot, resulting in distal thrombosis, may occur (Khoshmohabat, Paydar, Kazemi and Dalfardi, 2016).
4) List of abbreviation was added.

Reviewer # 4

1) According to the reviewer’s comment, a discussion about interaction of CH with GAG was added as follows: “As reported in literature, structure and stability of PECs strongly depend on different parameters such as concentrations of each polyelectrolyte, charge molar ratio, charge density, molar masses and chain flexibility but depend also on extrinsic parameters as pH, ionic strength and/or temperature (Kabanov, 2005; Feng, Leduc, and Pelton, 2008; Le Cerf, D., Pepin, A. S., Niang, P.M., Cristea et al., 2014).
   According to all these parameters, mixing oppositely charged polyelectrolytes could give two types of PECs dependently of charge stoichiometry: soluble PECs, leading to stable and transparent solutions, and insoluble PECs, with or without precipitation. The soluble PECs are obtained with an excess of anionic or cationic charges (Feng, Leduc, and Pelton, 2008; Kabanov and Zezin, 1984; Schatz, Domard, Viton, Pichot, et al., 2004) and this was the case of CH-HA and CH-CS. In fact, charge density (positive) is 0.006 mole/g for chitosan, 0.0013 mole/g for HA (negative) and 0.002 mole/g for CS (negative) with a clear excess of positive charges, in both cases. The charge molar ratio of CH – HA was 4.6 while that of CH – CS was 3.
   Moreover Denunziere, Ferrier, and Domard (1996) reported interaction between CS and CH was independent of CS sulfate substitution on 4 or 6 carbons.”

Acetic acid titration and zeta potential were aldo performed and the results commented as follows:
This was confirmed by zeta potential evaluation. CH 1 had a positive zeta potential of 53.2±1.9 (mean value±sd; n=3) while HA and CS as solutions showed negative zeta potentials of -27.9±0.9 and -16.8±1.2, respectively (mean value±sd; n=3). CH 1 – HA and CH 1 – CS were characterized by positive zeta potentials of 39.3±0.3 and 25.3±0.8, respectively (mean value±sd; n=3). These results supported that the interaction between CH and GAGs did not cause a neutralization of the two polyelectrolytes mixed (CH and HA or CS) and an excess of positive charges was present.
Moreover lyophilization caused acetic acid evaporation. In particular, acetic acid was added, to form chitosan acetate, in excess: 350 μmoli of acetic acid were added to 110 μmoli of chitosan per each dressing prepared, starting from chitosan at 1% w/w. The titration of acetic acid residues after lyophilization revealed that there was a residues of 5±1 μmoli per each systems independently of the addition of HA or CS. This is conceivably due to the almost total removal of acetic acid in excess (not involved in chitosan salification).

Moreover FT-IR analysis was performed and the results were commented as follows:
Figure 5 reports the FT-IR spectra of CH 1, CH 1 – HA and CH 1 – CS HA and CH+CS dressings and of CH powder (in the inset) as comparison.

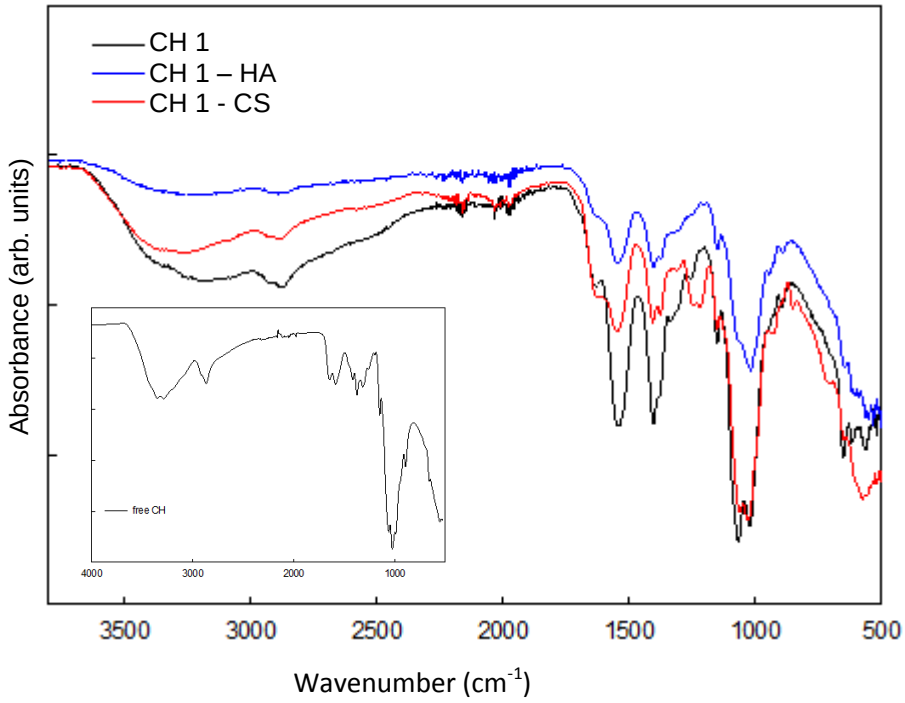


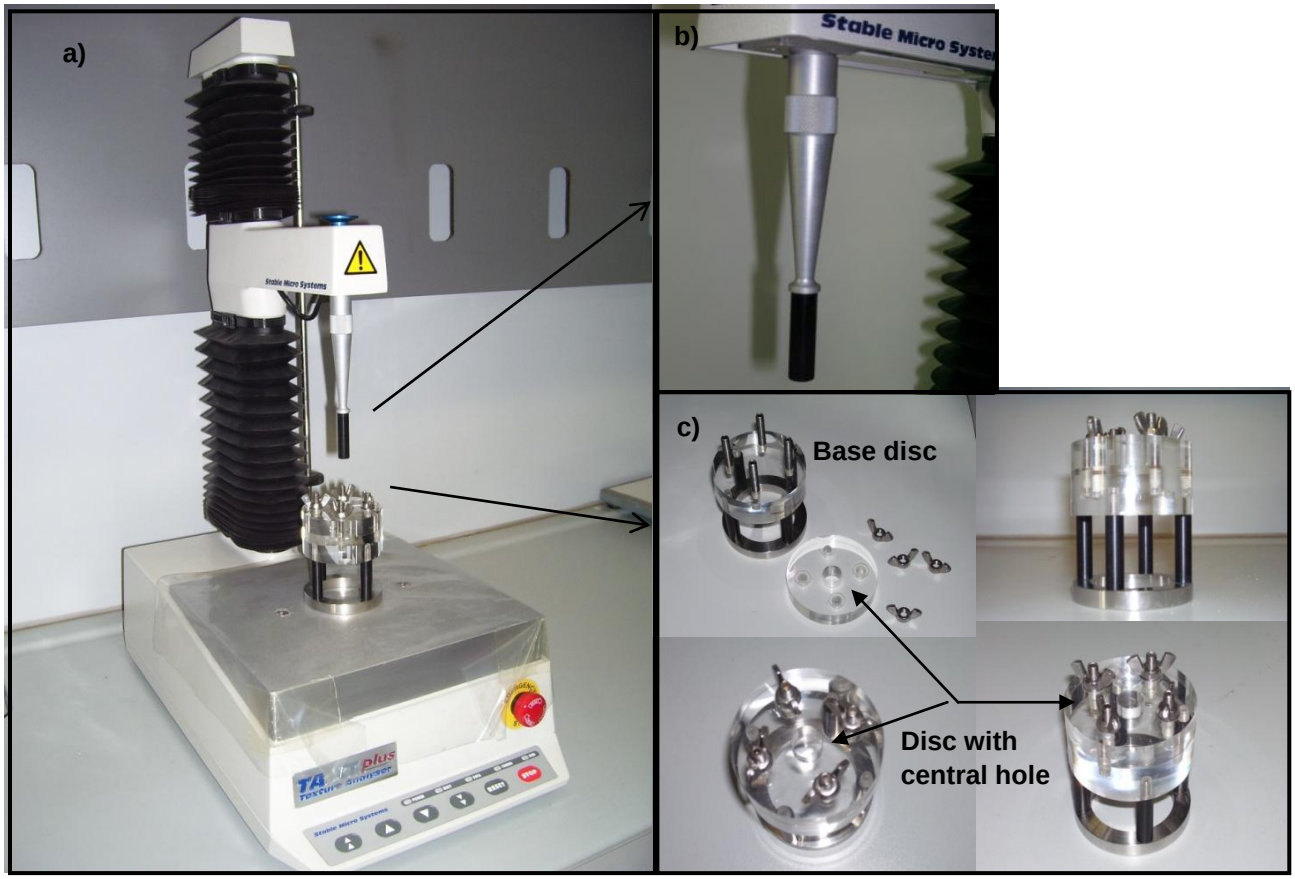
Figure 5: FT-IR spectra of CH 1, CH 1 – HA and CH 1 – CS HA and CH+CS dressings. In the inset the FTIR spectrum of CH powder (free CH) is reported as comparison.

The three spectra shared many common features both in the number and position of the peaks and seemed to be dominated by the FT-IR pattern of CH (free CH as powder), where the region around 1630 and 1540 cm^{-1} is typical of the amide I and amide II stretching and bending modes (Radhakumary, Antony, Sreenivasan, 2011; Jayakumar, Prabhakaran, Nair, Tamura, 2010). Interestingly, the position of these peaks shifted to lower frequency with respect to free CH (i.e. not interacting with acetate ions) shown Figure 1 inset. In particular, the –C=O stretching of the amide I in free CH moved from 1652 to 1634 cm^{-1} when it interacted with acetate ions and it remained in the same position even in the CH 1 - HA and CH 1 - CS systems. Moreover, a new peak around 1404 cm^{-1} (correlated to the –C-H- bending) appeared in the spectra of CH 1 dressing with respect to free CH and was found also in the spectra of CH 1 - HA and CH 1 - CS. The two mentioned spectral features have been correlated to charge-charge neutralization (salification) which most probably occurred between CH and acetate ions and dominated the spectra of all the three systems shown in Figure 1 (Shanti Krishna, Radhakumary, Sreenivasan, 2015).
This suggests that, in presence of acetate ions, the charge-charge interaction, involving chitosan amino groups, was preferentially between CH and acetate and therefore the further addition of HA

and CS mostly interact with chitosan through less strong bonding such as polar interactions. Such results are in agreement with zeta potential values determined.

2) In the experimental part the hydration measurement procedure was described better to clarify the contact between the liquid and the sample:
"Dressings were placed on a 0.45 µm membrane (HA, Millipore, I) that covered a container filled with pH 7.2 phosphate buffer (USP), simulating wound exudate. The liquid could freely cross the membrane dependently of the capability of each system to absorb liquid. At dry state (before the beginning if the hydration test) and at prefixed times during hydration (after the contact between each dressing and the membrane), the dressings were weighted and the amount of liquid absorbed was normalized by the weight of the dried dressing."

3) It was not possible to include the picture of the instrument in the text because the restricted number of figured permitted (8 including also tables).
Hereafter you can fin the picture of the instrument and the measuring systems. This was published in a review of ours "Sandri, G., Rossi, S., Bonferoni, M. C., Ferrari, F., Mori, M., Caramella, C. (2012) The role of chitosan as a mucoadhesive agent in mucosal drug delivery. *Journal of Drug Delivery Science and Technology*, 21, 275-284" and this was cited in the text.



Picture of texture analyzer apparatus (a), consisting of probe (b) and measuring system A/MUC (c) (Sandri, Rossi, Bonferoni, Ferrari et al., 2012, with permission)

4) Figure 3 blank meaning was inserted in the experimental part as follows: Blank measurements were performed by using a filter paper disc wetted with 100 µl of isotonic saline solution (NaCl 0.9% w/v) instead of biological substrate: this allowed to evaluate cohesive properties of the sample.
5) The name of the samples was checked and rendered consistent in all the figures.
6) The in vitro "In vitro dynamic whole-blood clotting" paragraph was partially rewritten to better explain the results obtained.
"Figure 7 shows % hemoglobin absorbance profiles as function of time evaluated for a) unloaded dressings and b) TA loaded formulations.
A higher degree of hemoglobin absorbance indicates a slower clot formation rate.
Whole blood clotting formation was relatively slow and the hemoglobin absorbance reached the minimum level after about 420 s. A faster clot formation occurred in presence of all the dressings and

30 s immediately after the contact between blood and formulations the % of free hemoglobin was around 20-50%. The minimum level of hemoglobin absorbance was reached after about 300 s. Chitosan capability to bind and aggregate platelets causing agglutination of erythrocytes and activation of hemostasis was predominant in all the formulation considered also in presence of GAG.
TA is a synthetic analogue of lysine and its antifibrinolytic activity is related to reversible bindings with four to five lysine receptor sites on plasminogen or plasmin: this prevents plasmin from binding to and degrading fibrin and preserves clot structure. TA showed a synergic activity with chitosan in enhancing clot formation: all the formulations reached the minimum in hemoglobin absorbance in about 180 s, presenting a better clotting performance with respect to unloaded dressings. TA CH 1 dressing allowed to maintain a % of hemoglobin at 420 s not significantly different from initial value, while CH 1 dressing determined a significant increase in % of hemoglobin comparing the initial value with respect to the final one (at 420 s). TA CH1 –HA and TA CH1- CS dressings proved to maintain clot integrity stable from the beginning up to 420 s, while the same compositions unloaded showed a partial clot modification at 90 s with a sharp increase in % of hemoglobin.
The presence of GAG slightly decreased the procoagulant activity of the dressings. In fact, HA and CS are reported in literature as anticoagulant materials. Fragments of HA having a molecular weight lower that 500 kDa, as it is the case, cause clot structure susceptible to mechanical deformations and softening (Komorowicz, Balazs, Varga, Szabo, et al., 2016). CS is a sulfated glycosaminoglycan with heparin-like activity (Pandolfi, M., Hedner, U., 1984). On this basis, the procogulant acitivity of the systems seems exclusively due to chitosan and its synergic effect with TA."

7) Figure 7 was deleted.

Freeze dried chitosan acetate dressings with glycosaminoglycans and traxenamic acid

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Abstract

Bleeding control plays an important role to increase survival in the early phase after a traumatic event. The aim of the present work was the development of hemostatic sponge-like dressings based on chitosan, in association with glycosaminoglycans (GAG as chondroitin sulfate or hyaluronic acid) and the improvement of their hemostatic performance by loading tranexamic acid (TA). The dressings were prepared by lyophilizazion and were characterized for mechanical, hydration, bioadhesion properties and morphology. Moreover FTIR analysis was performed to understand the interactions bet ween the different polyelectrolytes present in the dressing. Clotting was investigated in vitro by using rat whole blood. Moreover in vitro biocompatibility and proliferation were evaluated towards fibroblasts. Ex vivo proliferation properties were assessed by using human skin.

All the dressings were characterised by mechanical, hydration and bioadhesion properties suitable to be applied on bleeding wounds and to absorb bleeding or wound exudate, avoiding tissue dehydration. TA release was fast and TA and chitosan showed a synergic effect to speed up clotting. The dressings were biocompatible and able to sustain cell proliferation in vitro and ex vivo in human epidermis. In conclusion, sponge-like dressings based on chitosan and GAG and loaded with TA are an effective tool to enhance hemostasis and healing in bleeding wounds.

Highlights

Chitosan and glycosaminoglycan dressings are characterized by mechanical, hydration and bioadhesive properties suitable to be applied on bleeding wounds.

Chitosan and glycosaminoglycan interaction was studied by FTIR and dressing morphology was characterized by SEM.

Chitosan and tranexamic acid have a synergic effect in clot formation.

Keywords: chitosan, glycosaminoglycans, tranexamic acid, hemostasis, wound healing

61 Abbreviations

62 CH: chitosan

63 GAGs: glycosaminoglycans

64 ECM: extracellular matrix

65 HA: hyaluronic acid

66 CVS: chondroitin sulfate

67 TA: tranexamic acid

68 GM: growth medium

69 g: glycerol

70 m: mannitol

71 I_s: absorbance of sample

72 I_r: absorbance of reference value

73 NHDFs: normal human dermal fibroblasts from juvenile foreskin

74 FCS: foetal calf serum

75 MTT: (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)

76 PEC: polyelectrolyte complex

1. Introduction

Since 2001 (at the beginning of Afghanistan and Iraq military conflicts), many medical advances in military trauma care have been made to decrease morbidity and mortality and in particular, efforts have been made to control internal and external hemorrhage. However massive bleeding remains the leading cause of combat death and the second leading cause of death after traumatic brain injury in the civilian sector (Bennet, 2017; Champion, Bellamy, Roberts and Leppaniemi, 2003). Moreover in surgery, the reduction of bleeding can positively influence patient's prognosis (Li et al., 2016). Thus, bleeding control plays an important role not only to increase survival but also to prevent the development of multiple organ failure in the early phase after a traumatic event (Shields and Crowley, 2014).

Advanced topical hemostatic devices (including bandages, fibrin glue, liquids, powders, gels, and scaffolds – generally referred to as dressings) have been developed to control hemorrhages, to reduce mortality and prevent further complications (Seon, Lee, Kwon, Kim, et al., 2017). These dressings can be grouped into three classes by mechanism of action: including blood factor concentrators, which concentrate blood cellular and protein components to promote clot formation, bioadhesive agents having strong adherence to tissues and, procoagulant supplementary, which provide local concentration of procoagulat blood factors (Granville-Chapman, Jacobs, and Midwinter, 2011). Furthermore, dressings cover wounds preventing risk of infections (Seon et al., 2017).

Many types of natural polysaccharides have proposed as dressing biomaterials. In particular, chitosan (CH), a linear cationic polysaccharide based on glucosamine and N-acetyl glucosamine, obtained by partial chitin deacetylation, is well known as hemostatic agent. CH is able to enhance coagulation, by both hemagglutination and promoting platelet activation. In presence of blood, CH forms a solid clot due to thrombin and platelet activation, fibrin polymerization and fibrin fibers cross linking by FXIIIa (Marchand, Rivard, Sun, and Hoemann; 2009; Iliescu, Hoemann, Shive, Chenite et al., 2008) and CH is able to stabilize clot structure preventing its lysis (Hoemann et al., 2007). Moreover, it possess other crucial properties fundamental in wound healing: it is biodegradable and characterized by antimicrobial properties. Furthermore, CH supports extracellular matrix regeneration stimulating granulation tissue formation, activating fibroblasts (Francesko and Tzanov, 2011) and enhancing their proliferation and migration (Sandri et al., 2011) in the early phases of healing. Moreover, it is able to enhance remodeling process increasing also angiogenesis.

Glycosaminoglycans (GAGs) play a crucial role in different stages of skin tissue regeneration and maturation, being important components of its extracellular matrix (ECM). Moreover, GAGs are able to bind proteins including several chemokines and growth factors. The polysaccharidic structure of glycosaminoglycans as well as the presence, position and number of sulfate groups within the polymer chain plays an important role on these GAG-protein interactions. Among glycosaminoglycans, chondroitin sulfate (CS) and hyaluronic acid (HA), are naturally present in mammals and have an important role in the healing process.

CS is a sulfated polysaccharide based on N-acetylgalactosamine-glucuronic acid disaccharide units. It is able to interact with different important positively charged biological molecules, such as growth factors, chemokines, cytokines and adhesion molecules, involved in tissue healing and it prolongs their activities by charge-charge interactions, avoiding their enzymatic degradation (Silbert and Sugumaran, 2002; Yamada and Sugahara, 2008). HA is a linear polysaccharide composed of a dimeric repeating unit of D-glucuronic

acid and N-acetyl-D-glucosamine and, during the tissue injuries, it takes part to the wound healing and coagulation process forming, together with fibrin, a matrix support for fibroblast migration and proliferation (Chen and Abatangelo, 1999; Jiang, Liang and Noble, 2007).

The polysaccharides based systems should be more biocompatible rather than protein ones. In particular Collagen, one of the major components of ECM seems the best candidate to obtain dressings/scaffolds for wound reparation but in literature it is reported as immunogenic material due to possible helical- recognition by antibodies, due to 3D intact triple helix conformation, peculiar aminoacid sequence able to start antibody recognition, presence of non-helical terminal regions (telopeptides) (Lynn, Yannas and Bonfield, 2004; Olsen, Yang, Bodo, Chang et al., 2003). Moreover it could have concerns of species-to-species transmissible diseases (xenozoonoses) (Cataldo, Ursini, Lilla and Angelini, 2008).

Given this premises, the aim of the present work was the development of hemostatic sponge-like dressings based on chitosan (CH), in association with chondroitin sulfate (CS) and hyaluronic acid (HA) and loaded with tranexamic acid (TA), to be used in case of massive bleeding.

Tranexamic acid (TA) is a synthetic derivative of the aminoacid lysine and acts as antifibrinolytic agent by reversibly blocking lysine sites on plasminogen molecules (Dunn and Goa, 1999). In particular, TA is able to inhibit fibrinolysis by displacing plasminogen from fibrin and by avoiding its degradation (Ker, Edwards, Rerei, Shakur and Roberts, 2012).

Sponge-like dressings should have advantages in application with respect to powdery hemostatic agents, especially in the treatment of intra-abdominal solid organ bleedings or injuries: if hemostatic agents are based on granules (microparticles) can leave residue in the lumen of the vessel and may occlude distal arterial flow. Moreover intraluminal dissemination of the clot, resulting in distal thrombosis, may occur (Khoshmohabat, Paydar, Kazemi and Dalfardi, 2016).

TA loaded sponge-like dressings were prepared by freeze-drying and were characterized by hydration, mechanical, bioadhesion properties and morphology. Moreover, FTIR analysis was performed to understand the interactions bet ween the different polyelectrolytes present in the dressing. The release profile of TA was evaluated in isotonic solution (NaCl 0.9%) by using Franz diffusion cells. The hemostatic performance of the systems was evaluated by means of a dynamic whole-blood clotting test. Furthermore, in vitro cytocompatibility and proliferation tests were assessed by using NHDFs (normal human dermal fibroblasts from juvenile foreskin). Finally, proliferation properties of TA loaded sponge-like dressings were evaluated by means of an ex-vivo test on human skin.

2. Experimental part

2.1. Materials

The following materials were used: Chitosan (CH) low MW 251000 Da, deacetylation degree 98%, maximum charge density: number of positively charged functional groups per repeated unit: 0.98 (ChitoClear, Siiiglufjordur-Iceland); Hyaluronic Acid (HA) low MW 212000 Da; maximum charge density: number of negatively charged functional groups per repeated unit: 0.5 (Bioiberica, Barenz, Italy); Chondroitin sodium sulfate bovine 100 EP (CS) low MW 14000 Da, mixture of A (chondroitin 4 sulfate) and C (chondroitin 6 sulfate); maximum charge density: number of negatively charged functional groups per repeated unit: 1 (Bioiberica, Barenz, Italy); Trans-4 (amino-methyl) cyclohexanecarboxyilic acid (Sigma Aldrich, Milan-Italy); Glycerol (g) 30° Be (Carlo Erba, Italy); D-Mannitol (Fluka, France).

2.2. Methods

2.2.1. Sponge-like dressing preparation

CH was hydrated in acetic acid 1% w/w (glacial acetic acid, Sigma Aldrich, Italy) under gentle stirring at room temperature. Acetate was used as counterion to obtain chitosan salification in mild pH conditions and it was preferred to hydrochloric acid because chitosan fragmentation could occur in HCl environment also at low molarity (Sabnis, Block, 2000).

HA and CS were hydrated in distilled water under gentle stirring at room temperature. The two polymeric solutions were mixed 1:1 weight ratio to obtain polymeric mixtures. Systems containing CH alone were mixed 1:1 weight ratio with bidistilled water. Final acetic acid concentration in the polymeric systems was 0.5% w/w. CH 1 - HA and CH 1 - CS mixtures and CH 1, were subjected to zeta potential evaluation. Measurements were carried out at 25°C by means of a Malvern Zetasizer Nano ZS90 (Malvern Ltd., UK). HA and CS solutions, prepared in the same concentrations used for CH 1 - HA and CH 1 - CS mixtures but without CH were also tested.

In two series of preparations of CH 1 - HA and CH 1 - CS mixtures and CH 1, glycerol or mannitol were added as lyoprotectants. Polymeric solutions appeared transparent without visible precipitate. As for TA loaded systems drug was added to the polymeric mixture at 0.75% w/w. Two ml of each mixture were poured into each well of 12-well plate (well area 3.8 cm^2). In the TA loaded systems the drug was 4 mg/cm^2. All the polymeric solutions were frozen at -40°C overnight and freeze-dried (Heto 15, Analitica De Mori, I) for 24 h. All the dressings prepared were 5 mm thick (sd ± 0.52).

CH, HA and CS were at 5 mg/cm^2 concentration in CH 1 dressings; CH was at 10 mg/cm^2 concentration in CH 2 dressings, glycerol was at 1.3 mg/cm^2 while mannitol was at 10.5 mg/cm^2 .

Dressings were re-solubilized in distilled water to check the eventual residues of acetic acid (2 g of distilled water per each dressing). pH of each sample was measured by means of a pHmeter (pH 210 Microprocessor, Hanna Instruments, Italy) and the acetic acid excess was titrated by means of acid-base titration by using phenolphthatelin as indicator and NaOH 0.1 M (Carlo Erba.Italy).

2.2.2. Sponge-like dressing characterization

2.2.2.1. Penetrometry measurements

Sponge-like dressings were subjected to penetrometry measurements by means of a texture analyzer (TA.XT plus, Stable Microsystems, ENCO, Spinea, I), equipped with a cylinder probe (10 mm, P10), to evaluate the resistance to compression. Each dressing was placed onto the apparatus base and the probe was lowered at 1 mm/s speed. Maximum force of resistance to compression (Fmax) was determined as the maximum force required to penetrate into the sponge up to 2.5 mm.

2.2.2.2. Hydration measurements

Dressings were placed on a 0.45 µm membrane (HA, Millipore, I) that covered a container filled with pH 7.2 phosphate buffer (USP), simulating wound exudate. The liquid could freely cross the membrane dependently of the capability of each system to absorb liquid. At dry state (before the beginning if the hydration test) and at prefixed times during hydration (after the contact between each dressing and the membrane), the

dressings were weighted and the amount of liquid absorbed was normalized by the weight of the dried dressing.

2.2.2.4. Bioadhesion measurements

Bioadhesion measurements were performed using texture analyzer (TA.XT plus, Stable Microsystems, ENCO, Spinea, I) equipped with a 1 kg load cell, a cylinder probe having a diameter of 10 mm (P10) and the measuring system A/MUC (adhesion test system) (Sandri, Bonferoni, D'Autilia, Rossi et al., 2013; Szucs, Sandri, Bonferoni, Caramella et al., 2008). The measuring system A/MUC consists of a support in which a biological substrate can be fixed. In this case, the biological substrate was egg shell membrane (Tao, Lu, Sun, Gu et al., 2009), chosen to mimic damaged skin, wetted with 100 µl of isotonic saline solution (NaCl 0.9% w/v). To obtain the membrane, the egg shell was placed in a 0.5 M HCl solution for 1 h. Sponge like dressings, having diameter of 10 mm, were stuck with cyanoacrylate glue to the cylinder probe and hydrated with 100 µl of pH 7.2 phosphate buffer (USP). The sample and the biological substrate were put in contact under a preload of 0.02 N for 3 min. The cylinder probe was then moved upward at a prefixed speed of 2.5 mm/s up to the complete separation of the bioadhesive interface (egg shell membrane-sample). The maximum force of detachment (Fmax) was recorded as a function of displacement.

Blank measurements were performed by using a filter paper disc wetted with 100 µl of isotonic saline solution (NaCl 0.9% w/v) instead of biological substrate: this allowed to evaluate cohesive properties of the sample. As comparison, two physical mixtures (CH-HApm and CH-CSpm) having the same polymer composition than sponge-like dressings were evaluate. Moreover the bioadhesion of CS and HA as powders (CSp and HAp) were compared to understand the contribution of GAG to bioadhesion. In these latter cases, the same experimental conditions (hydration and sample weight) were used.

2.2.2.5. Morphology

Dressing morphology was analyzed by means of scanning electron microscopy (SEM, Tescan, Mira3XMU, ARVEDI Center, University of Pavia). Samples were sputtered by means of graphite deposition under vacuum.

2.2.2.6. FT-IR measurements

FT-IR spectra were obtained using a Nicolet FT-IR iS10 Spectrometer (Nicolet, Madison, WI, USA) equipped with ATR (Attenuated Total Reflectance) sampling accessory (Smart iTR with ZnSe plate) by co-adding 256 scans in the 4000–650 cm^{-1} range at 4 cm^{-1} resolution.

2.2.2.7. TA release measurements

TA release measurements were performed in vitro by using Franz cells (vertical glass diffusion cell, orifice diameter 20 mm, PermeGear Inc., USA). A cellulose acetate membrane (pore size 0.45 µm, diameter 25 mm) was placed between the donor and the receptor chambers. The cells were thermoset at 32°C by means of a water jack. Isotonic solution (NaCl 0.9% w/v) was used as receiving phase. Each dressing was placed in the donor chamber and wetted with 100 µl of isotonic solution. At prefixed times, 500 µl of receiving phase were collected and the amount of drug released was assayed by means of a spectrophotometric method (Ansari, Raza, and Rehman, 2005). This method is based on the colorimetric detection of the Ruhemann's

purple resulting from ninhydrin and TA reaction via oxidation deamination of TA primary amino group, followed by the condensation of the reduced ninhydrin in the basic medium at pH 8.0. TA stock solution was prepared in NaCl 0.9 % w/v at 1 mg/ml concentration and a calibration curve was prepared with TA concentrations ranging from 1 mg/ml to 0.1 mg/ml. 1 ml of ninhydrin reagent (0.2 % w/w ninhydrin (Carlo Erba, Milan, I) in methanol) and 0.5 ml of 20 mM phosphate buffer at pH 8.0 were added to 200 μl of each sample. Each sample was heated at 90 °C for 20 min in a shaking bath, subsequently they were cooled at room temperature and their absorbance was read at 565 nm wavelength (Lamba 25, Perkin Elmer, I). The method was linear in the concentration range from 1 mg/ml to 0.1 mg/ml with R^2 higher than 0.995.

2.2.2.8. In vitro dynamic whole-blood clotting

The hemostatic activity of sponge-like dressings was evaluated using rat whole blood pooled from 6 male rats (Wistar 200–250 g). All animal experiments were carried out in full compliance with the standard international ethical guidelines (European Communities Council Directive 86/609/EEC) and approved by Italian Health Ministry (D.L. 116/92). The study protocol was approved by the Local Institutional Ethics Committee of the University of Pavia for the use of animals. The whole blood was supplemented with 10% v/v of acid-citrate-dextrose (ACDC, 38 mM citric acid/ 75 mM trisodum citrate /100 mM dextrose) to avoid the coagulation during the storage at 4°C (Quan, Lia, Luana, Yuana, et al., 2015; Ong, Wu, Moochhala, Tan, et al., 2008; Sudheesh Kumar, Lakshmanan, Anilkumar, Ramya, et al., 2012). Just before experiments, the anticoagulant activity of ACDC was inhibited by adding 100 µl of saturated $CaCl_2$ solution to 50 µl of blood.
50 µl volume of fresh blood was dropped onto unloaded and TA loaded sponge dressings (5 mm in diameter) placed in 50 ml glass beaker. After prefixed contact times, 10 ml distilled water was slowly poured in beaker without disturbing the clotted blood. The beaker was gently shaken for 2 minutes to suspend free red blood cells, not entrapped in the clot. The absorbance of each resulting sample, was assayed by means of spectrophotometric detection at 542 nm wavelength (Lamba 25, Perkin Elmer, I). As reference, fresh blood was considered.
The content of hemoglobin was quantified by the following equation:
Hemoglobin absorbance % = $I_s/I_r \times 100$
where I_s is the absorbance of the resulting sample, and I_r is the absorbance of the reference value (blood or blood/TA (Ong, Wu, Moochhala, Tan, et al., 2008; Sudheesh Kumar, Lakshmanan, Anilkumar, Ramya, et al., 2012).

2.2.2.9. In vitro biocompatibility and proliferation

NHDFs (normal human dermal fibroblasts from juvenile foreskin, Promocell GmbH, Heidelberg, G) were used between the 2^{nd} and 5^{th} passage, for all the experiments.
Fibroblasts were grown in presence of Dulbecco's modified Eagle medium (Sigma, I) and supplemented with 10% fetal calf serum (FCS, Euroclone, I) with 200 IU/ml penicillin, and with 0.2 mg/ml streptomycin (Sigma, I) and kept at 37° C in a 5% CO_2 atmosphere with 95% relative humidity (RH).
Fibroblasts were seeded in each well of 96-well plates (area 0.34 cm^2) at a seeding density of 10^5 cells/cm^2. Cells were grown 24 h to obtain sub-confluence.
3.5 x 10^4 cells/well (area 0.34 cm^2) were seeded in a 96-well plate and grown to confluence for 24 h.

After 24 h cells were washed with saline solution and placed in contact with 200 µl of CH, CH-CS and CH-HA polymeric mixtures (prepared as reported in the section 2.2.1.) at 1:20 (CH, CS or HA at 500 µg/ml, solubilized in cell growth medium) and 1:50 dilution with growth medium (GM) (CH, CS or HA at 200 µg/ml). After dilution, all the samples were transparent without visible precipitate. TA having the same concentration as in the loaded polymeric mixtures and GM were used as control. Cell substrates were incubated for 3 h (biocompatibility test) and for 24 h (proliferation test) with the samples, then the medium was removed and the MTT test was performed. Briefly, MTT test is based on the activity of mitochondrial dehydrogenases of vital cells that convert MTT in formazan (Sandri, Aguzzi, Rossi, Bonferoni et al., 2017). At this purpose, 50 µl of MTT solution (Sigma Aldrich, I) at 2.5 mg/ml concentration in HBSS (Hank's Buffered Salt Solution) pH 7.4 was put in contact with each cell substrate for 3 h. The reagent was removed from each well, and the substrates were washed with 200 µl of PBS. After the removal of PBS, 100 µl of DMSO was put in each well, and the absorbance was assayed at 570 nm by means of an ELISA plate reader (Imark Absorbance Reader, Biorad, I), with a reference wavelength of 690 nm. Cell viability was calculated as % ratio between the absorbance of each sample and the absorbance of cell substrate maintained in contact with growth medium.

2.2.2.10. Ex vivo proliferation on human skin biopsy

To analyze the effects of dressings on wound healing, an ex vivo human skin model was used to reproduce in vivo physiological conditions (Mori, Rossi, Ferrari, Bonferoni et al., 2016). Adult healthy human skin biopsies were clinically obtained from surgery for breast reduction of different age donors (range 35-60 years old), after obtaining informed consent. Surgical biopsy (about 4x8 cm) was cut into smaller fragments (about 0.6x0.6 cm) and a skin circular portion (including epidermis and dermis) was centrally removed with aseptic circular punch (diameter: 3 mm). Each fragment was placed into Transwell® inserts (growing area: 0.33 cm^2, Corning® Costar®, Sigma, I) in 24-well-plate (membrane pore size: 0.4 µm) and circular portions of each dressing, having the same diameter of punch, were applied in correspondence of skin lesion.

The samples were maintained in culture in presence of Dulbecco's modified Eagle medium (Sigma, I) and supplemented with 10% fetal calf serum (FCS, Euroclone, I) with 200 IU/ml penicillin, and with 0.2 mg/ml streptomycin (Sigma, I) and kept at 37° C in a 5% CO_2 atmosphere with 95% relative humidity (RH).

The medium (900 µl/each well) was added into well basal compartment to maintain skin viability, avoiding skin dehydration and ensuring an adequate supply of nutrients through hypodermis. After different times (24 and 72 h and 7 days) of culture, the biopsies were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 24 h, and processed for histological analysis (Riva, Casasco, Nespoli, Icaro Cornaglia, et al., 2007). Briefly, the fragments were dehydrated through graded concentrations of ethanol and embedded in paraffin. 8 µm sections were obtained by means of a microtome (Leitz, G), rehydrated and processed for immunohistochemical reactions for bromodeoxyuridine (BrdU) incorporation. The sections were incubated with primary antibody anti-BrdU (Ge Healthcare, UK) overnight at appropriate dilution (1:100), the primary antibody anti-BrdU was reacted by using MACH 1 Universal HRP-Polymer Detection kit (Biocare Medical, USA). At this purpose the sections were reacted with MACH 1 Mouse probe for 15 min and subsequently with HRP-Polymer (horseradish peroxidase polymer) for 30 min and finally with Biocare's Betazoid DAB for 5 min. Finally, the sections were dehydrated and mounted with DPX mounting medium (a mixture of distyrene, a plasticizer, dissolved in toluene-xylene, Sigma, I).

Skin sections obtained in the middle of the lesions were observed at the magnification of 20X under a light microscope Axiophot (Zeiss, G) equipped with a digital camera. Image J program was used to analyze images, counting cell nuclear positivity in a section area larger 2 mm at right and left side than the lesions.

2.2.2.11. Statistical analysis

Statistical differences were evaluated by means of a non-parametric test: Mann Whitney (Wilcolxon) W test, (Stat Graphics 5.0, Statistical Graphics Corporation, MD, USA). Differences were considered significant at $p<0.05$; only significant differences are evidenced in the figures as asterisks.

3. Results and discussion

3.1. Sponge-like dressing technological properties

3.1.1 Mechanical properties

Figure 1 reports the results of mechanical resistance as maximum force of penetration of the dressings without lyoprotectant and with glycerol or mannitol as lyoprotectants.

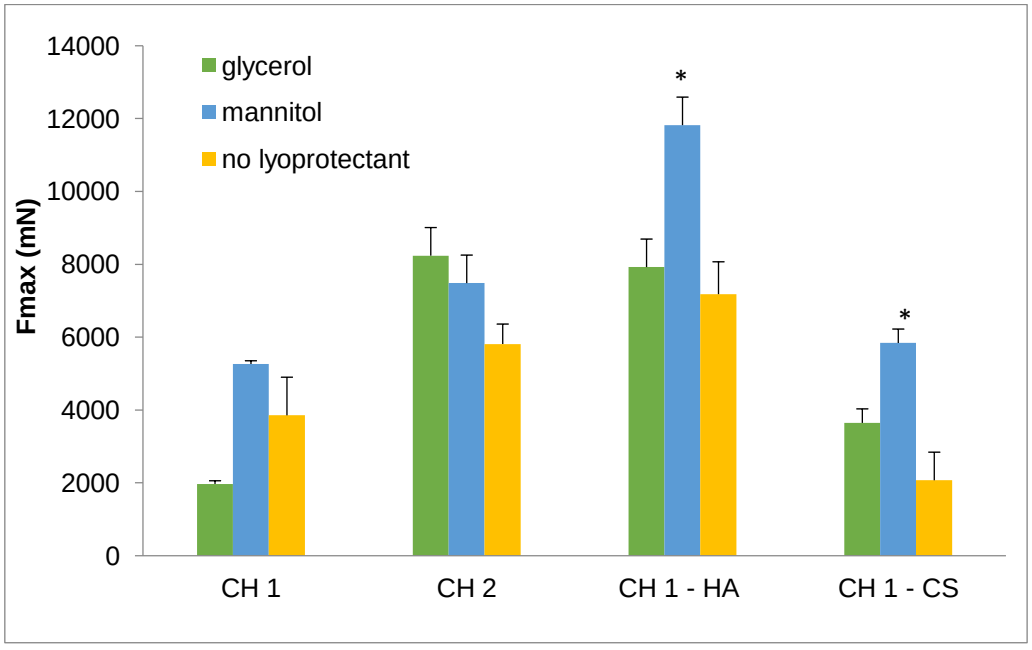


Figure 1: mechanical resistance as maximum force of penetration (Fmax, mN) of the dressings without lyoprotectant and with glycerol or mannitol as lyoprotectants (mean values±sd; n=6)

The increase of chitosan amounts in the dressings increased the mechanical resistance of the formulations: stiffness was significantly greater when lyoprotectants (glycerol or mannitol) were included in the formulations. The presence of HA in the dressings caused a significantly increase in the mechanical resistance and HA based formulation with mannitol, as lyoprotectant, was characterized by the higher Fmax value to indicate a more rigid structure. This behavior could probably due to the high molecular weight of HA, with respect to CS, that could dramatically strengthen the tridimensional network of the system. It is conceivable that HA interacted with chitosan during the preparation procedure, forming a polyelectrolyte

complex (PEC) between chitosan and hyaluronate. Since both CH and HA have similar molecular weight the interaction between the two polymers should be able to form a highly crosslinked structure.

On the contrary, the presence of CS in the formulation did not significantly change the stiffness of the dressings. This is probably due to the resulting PEC formed by an interaction between CS and CH: CS is characterized by a molecular weight 18 folds lower than that of CH, moreover sulfate groups of CS (more acid with respect to carboxylic group) could cause a coiled structure less prone to polymer chain entanglements. This probably rendered the dressing structure less stiff.

As reported in literature, structure and stability of PECs strongly depend on different parameters such as concentrations of each polyelectrolyte, charge molar ratio, charge density, molar masses and chain flexibility but depend also on extrinsic parameters as pH, ionic strength and/or temperature (Kabanov, 2005; Feng, Leduc, and Pelton, 2008; Le Cerf, D., Pepin, A. S., Niang, P.M., Cristea et al., 2014).

According to all these parameters, mixing oppositely charged polyelectrolytes could give two types of PECs dependently of charge stoichiometry: soluble PECs, leading to stable and transparent solutions, and insoluble PECs, with or without precipitation. The soluble PECs are obtained with an excess of anionic or cationic charges (Feng, Leduc, and Pelton, 2008; Kabanov and Zezin, 1984; Schatz, Domard, Viton, Pichot, et al., 2004) and this was the case of CH-HA and CH-CS. In fact, charge density (positive) is 0.006 mole/g for chitosan, 0.0013 mole/g for HA (negative) and 0.002 mole/g for CS (negative) with a clear excess of positive charges, in both cases. The charge molar ratio of CH – HA was 4.6 while that of CH – CS was 3.

Moreover Denunziere, Ferrier, and Domard (1996) reported interaction between CS and CH was independent of CS sulfate substitution on 4 or 6 carbons.

This was confirmed by zeta potential evaluation. CH 1 had a positive zeta potential of 53.2±1.9 (mean value±sd; n=3) while HA and CS as solutions showed negative zeta potentials of -27.9±0.9 and -16.8±1.2, respectively (mean value±sd; n=3). CH 1 – HA and CH 1 – CS were characterized by positive zeta potentials of 39.3±0.3 and 25.3±0.8, respectively (mean value±sd; n=3). These results supported that the interaction between CH and GAGs did not cause a neutralization of the two polyelectrolytes mixed (CH and HA or CS) and an excess of positive charges was present.

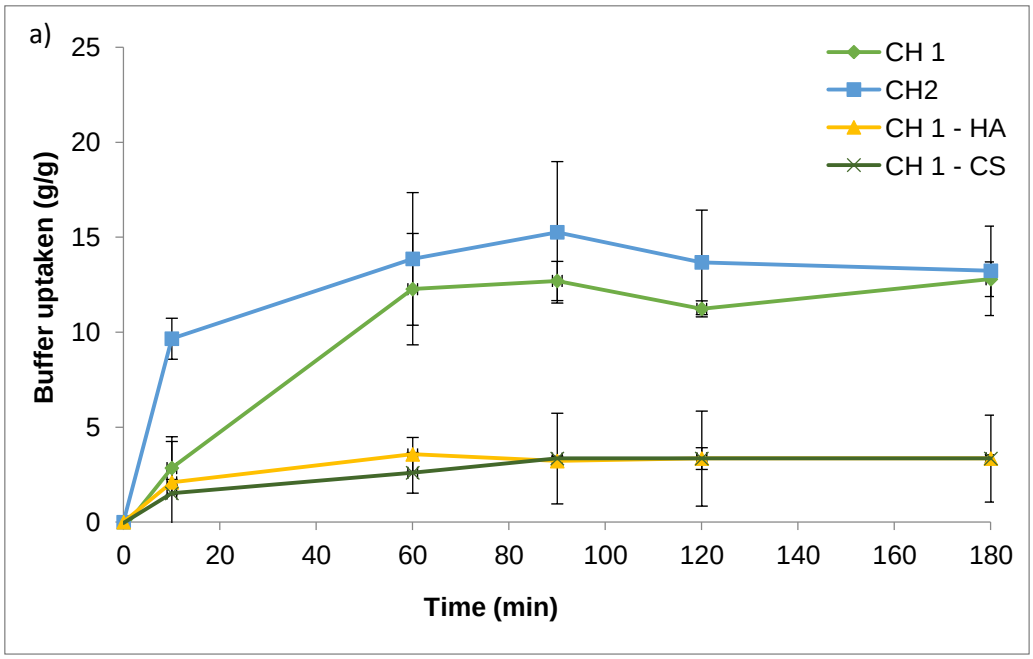
Moreover lyophilization caused acetic acid evaporation. In particular, acetic acid was added, to form chitosan acetate, in excess: 350 μmoli of acetic acid were added to 110 μmoli of chitosan per each dressing prepared, starting from chitosan at 1% w/w. The titration of acetic acid residues after lyophilization revealed that there was a residues of 5±1 μmoli per each systems independently of the addition of HA or CS. This is conceivably due to the almost total removal of acetic acid in excess (not involved in chitosan salification).

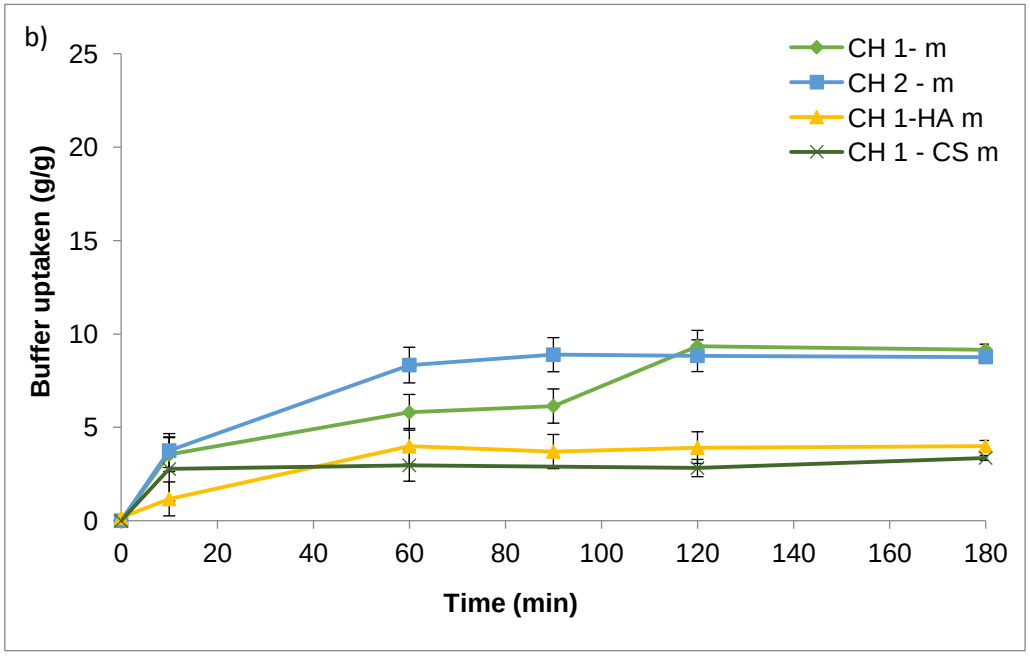
3.1.2. Hydration properties

Figure 2 reports buffer (pH 7.2 phosphate buffer, to mimic wound exudates) taken up a function of time for all the dressings prepared: a) without lyoprotectant, b) with glycerol and c) with mannitol as lyoprotectans.

Considering all the compositions with and without lyoprotectants, chitosan based dressings were characterized by higher buffer taken up vs time profile with respect to those of chitosan and GAG systems; moreover chitosan dressings based on higher CH amounts, showed higher hydration profile even not significantly different from hydration capability of dressing based on lower CH amounts. Dressings based on chitosan and GAG were characterized by significantly lower hydration profiles, independently of both the presence and type of lyoprotectants. All the systems showed a high hydration capacity in the first hour: it is

conceivable that chitosan was likely to create a lower pH environment at the dressing/liquid interface and this caused a high liquid absorption probably due chitosan gelation. Subsequently, chitosan poor solubility at neutral pH (pKa 6.5) prevailed over its buffering properties and liquid absorption stopped. The presence of GAG further decreased system hydration probably due to the interpolymer interaction between chitosan and hyaluronic acid or chondroitin sulfate.





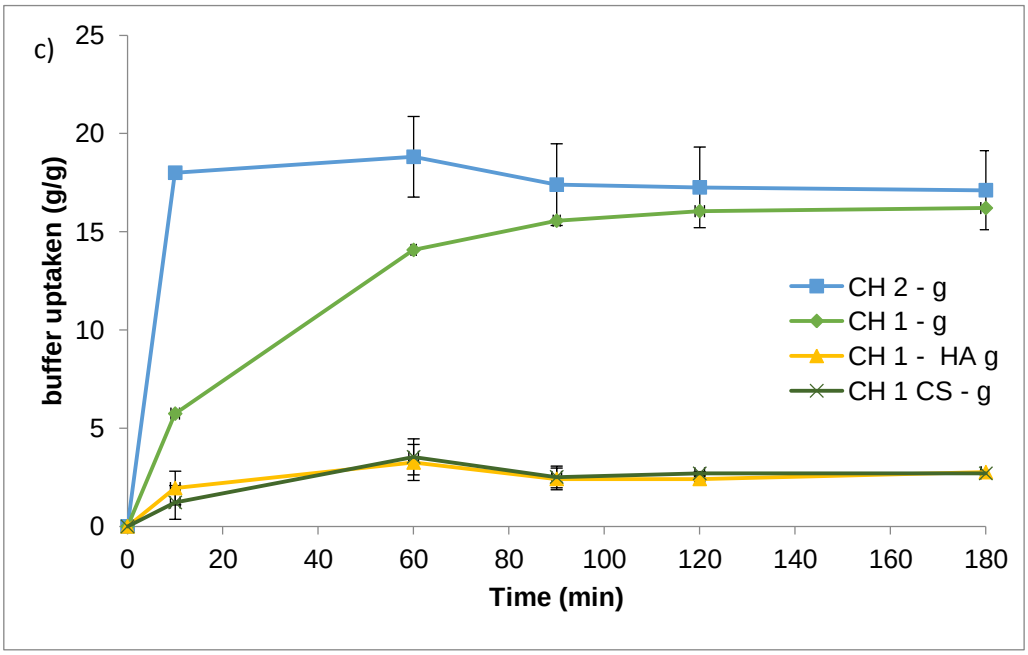
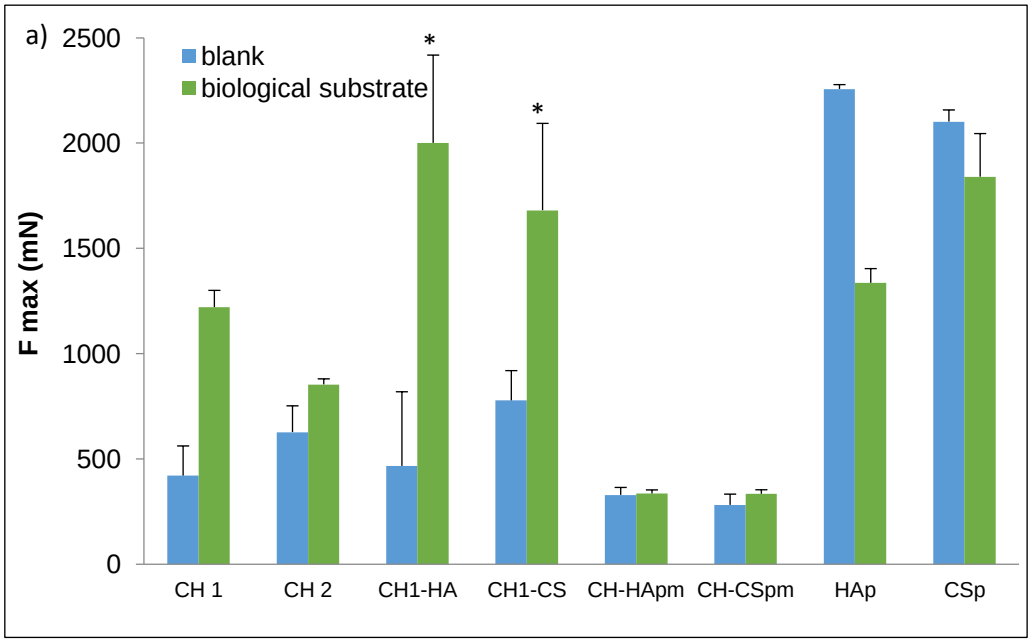


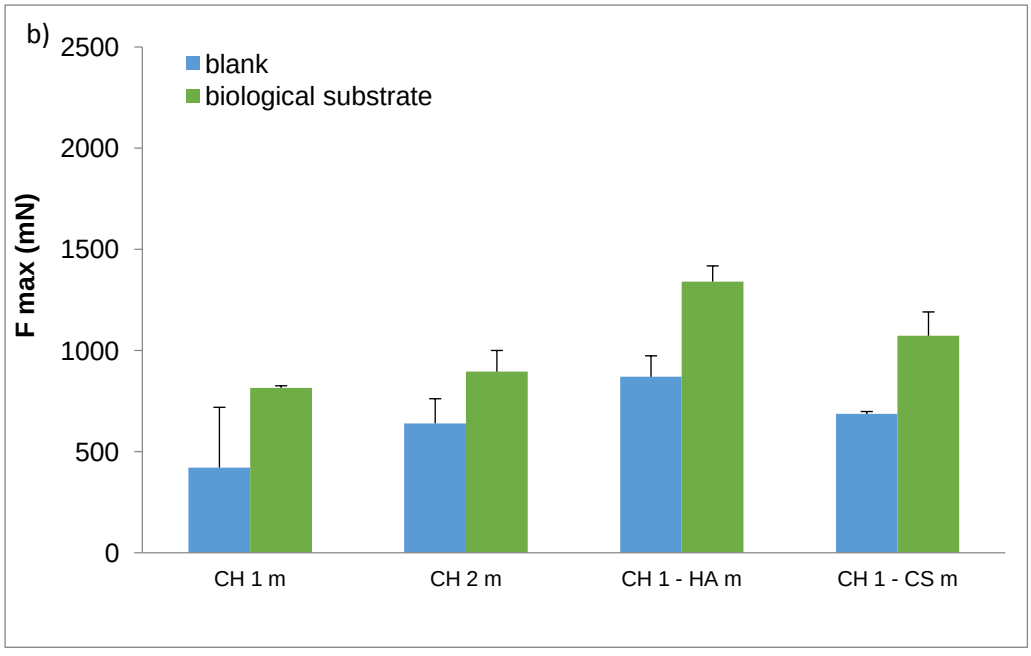
Figure 2: liquid taken up (pH 7.2 phosphate buffer) as function of time for all the dressings prepared: a) without lyoprotectant, b) with mannitol and c) with glycerol as lyoprotectans (mean values±sd; n=6)

Glycerol did not markedly change the hydration properties of CH and CH in association with GAG. On the contrary, mannitol substantially decreased the hydration capacity of the systems: this could be related to stiff structure of the dressings, which could slow down and impair liquid penetration into the dressings.
Hydration behavior is a crucial point in dressings to ensure hemostasis, to absorb wound exudate and to avoid wound bed dehydration, enhancing granulation phase and healing.

3.1.3. Bioadhesion properties

Figure 3 reports maximum force of bioadhesion (mN) all the dressings prepared: a) without lyoprotectant, b) with glycerol and c) with mannitol as lyoprotectans.
As for dressings without lyoprotectants (Fig. 3 a), chitosan concentration influenced bioadhesive behavior: CH 2 sponge-like dressing (containing higher chitosan amount) did not show bioadhesive propensity (Fmax measured without biological substrate was not significantly different with respect to the value in presence of biological substrate – egg shell membrane) while CH 1 dressing (containing chitosan at lower amount) was characterized by good bioadhesive properties. Such a behavior is probably due to a different degree of entanglement between chitosan polymeric chains. In particular, the lowest chitosan concentration the lowest the entanglement and the highest the capability to form a bioadhesive joint with the biological substrate (Rossi, Ferrari, Bonferoni, Caramella, 2001).
Since zeta potential analysis confirmed that there was not a complete neutralization of CH with GAGs and both CH-HA and CH-CS had positive zeta potentials, the presence of GAG in dressings significantly enhanced the bioadhesive properties. The interaction between chitosan and GAG probably assisted bioadhesive joint consolidation, considering a higher concentration of carboxylic and OH residues in these systems with respect to that of CH 1. The highest presence of these groups able to form hydrogen bonds could consolidate bioadhesive properties of chitosan, that are mainly due to its positive charge density.
Chitosan and GAG physical mixtures (CH-HApm, and CH-CSpm) did not show bioadhesive properties: these are easily inferred by the negligible solubility properties of chitosan at pH 7.2 phosphate buffer (hydration medium chosen to mimic wound bed exudate). Moreover CS or HA, as powders, were also considered and they showed a lack of bioadhesion in those conditions: this was probably due to slower hydration of HA during the test with respect to the lyophilized dressings and to the poor bioadhesive strenght of CS mainly caused by its low molecular weight.
In presence of lyoprotectans, both mannitol and glycerol (Fig. 3 b and c, respectively), the bioadhesive properties were less pronounced and were significant only for HA and CS dressings with mannitol. The presence of lyoprotectans, especially of mannitol, that increased system stiffness and probably decreased polymer chain mobility, could impair polymer capability to interact with the biological substrate.
Also bioadhesion is a crucial characteristic that should favor an intimate and prolonged contact between wound dressings and lesion, favoring blood absorption and avoiding formulation detachment to increase the hemostatic potential.


a)
2500
2000
1500
1000
500
0
F max (mN)
blank
biological substrate
*
*
CH 1
CH 2
CH1-HA
CH1-CS
CH-HApm
CH-CSpm
HAp
CSp


b)
2500
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biological substrate
CH 1 m
CH 2 m
CH 1 - HA m
CH 1 - CS m

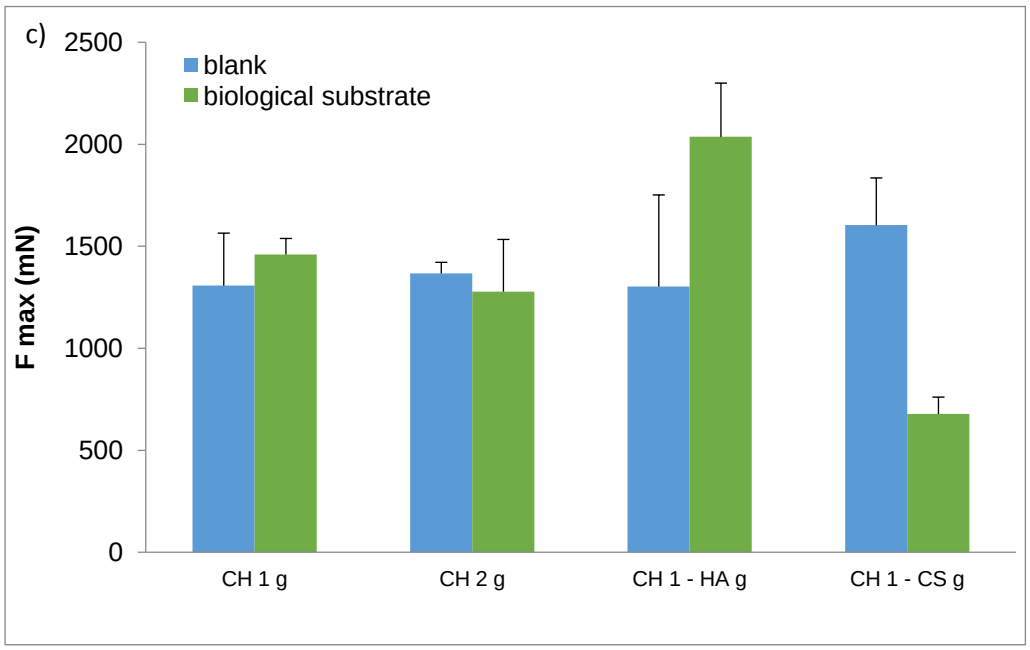

Figure 3: maximum force of bioadhesion, Fmax (mN) of all the dressings prepared: a) without lyoprotectant, b) with glycerol and c) with mannitol as lyoprotectans (mean values±sd; n=6)

3.1.4. Morphology

To understand if the system tridimensional networks could affect hydration and bioadhesion properties, morphology was assessed.

Figure 4 reports SEM images of transversal sections of CH 1, CH 1- HA and CH 1 – CS unloaded dressings and TA loaded dressings without lyoprotectans, and CH 1, CH 1- HA and CH 1 – CS dressings with glycerol and with mannitol.

CH 1 dressing showed a beehive structure with polyhedric cavities interconnected by pores having oval or round shapes. The cavities had dimensions of about 500 μm while the pores ranged from 50 to 200 μm. The presence of GAG did not change the system morphology while it markedly decreased the cavity dimensions that were around 200-300 μm and furthermore the pore diameters that were of about 50 μm. This difference in system porosity conceivably contributed to their hydration behavior. The TA loading did not modify the structure of the dressings.

The presence of glycerol as lyoprotectant did not substantially change dressing structure while the presence of mannitol caused a partially loss of polyhedric cavities and the structure resembled much more randomly organized sheets without marked modifications of the porosity. This modification could be responsible to the system behavior upon hydration and bioadhesion.

Since lyoprotectans did not markedly improve the technological properties of the dressings and considering mechanical, hydration and bioadhesive properties, lyoprotectant free systems prepared using chitosan at 1% w/w were considered for the further characterizations.

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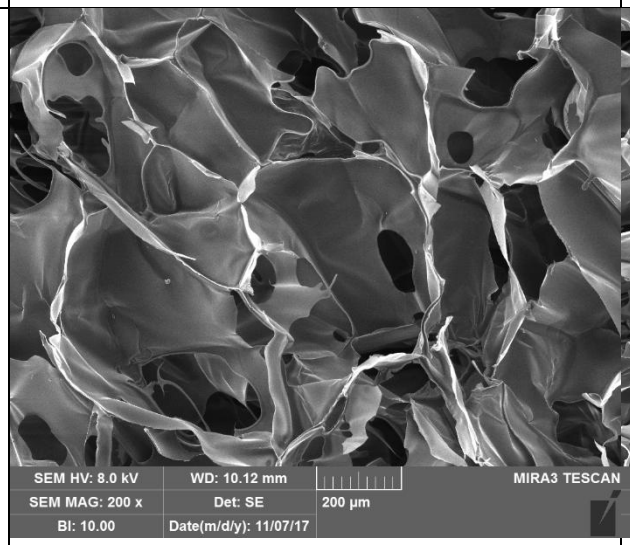
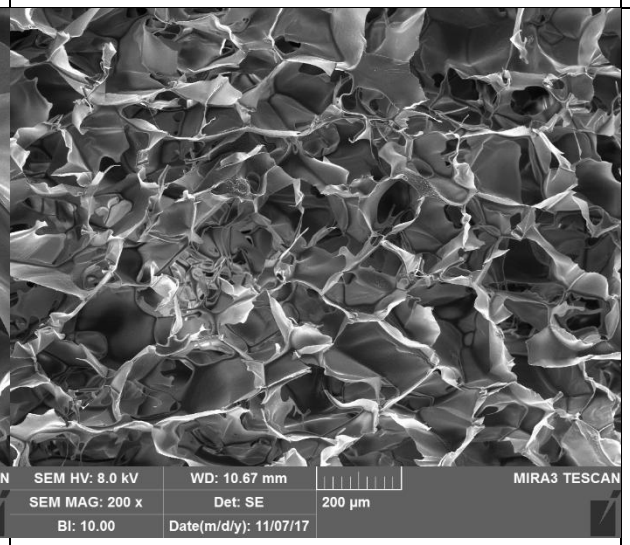
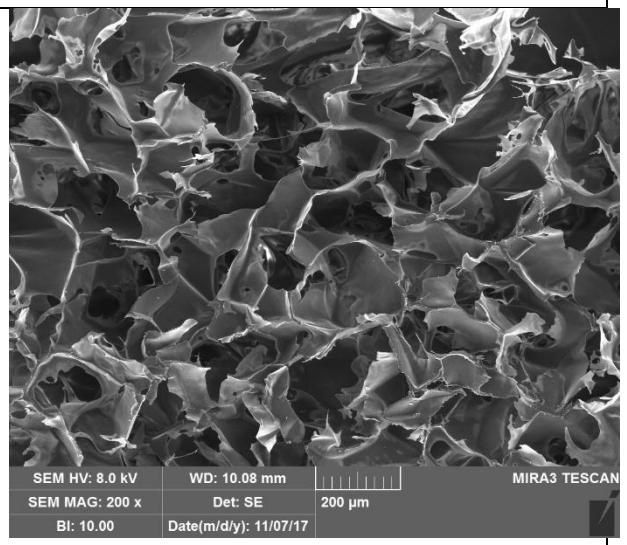
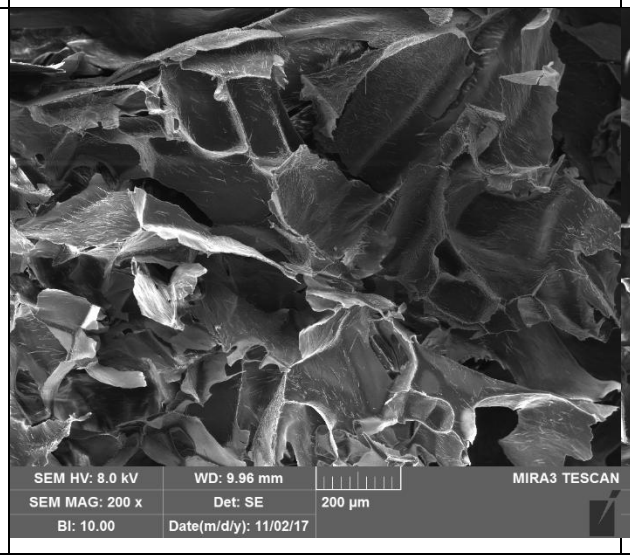
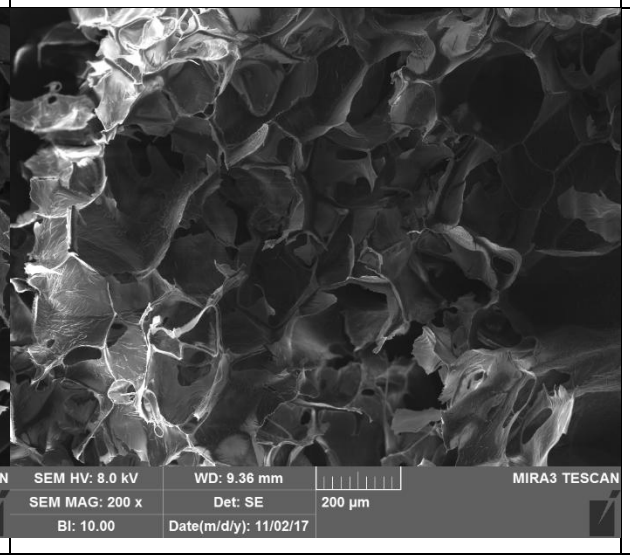
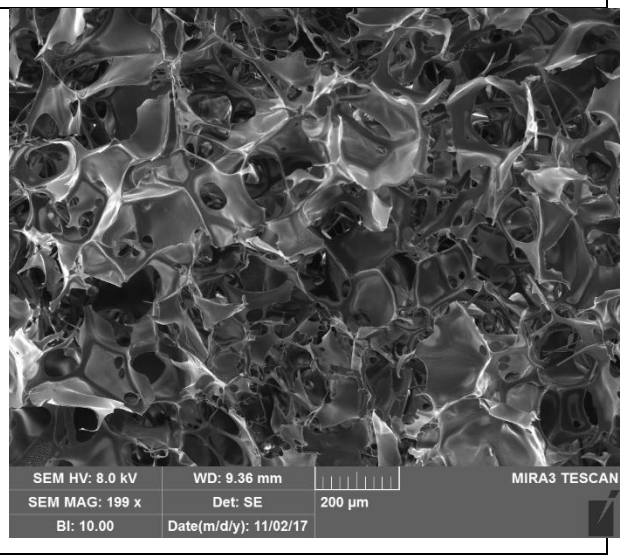
	CH 1	CH 1 - HA	CH1 - CS
No lyoprotectant			
TA loaded dressing without lyoprotectant			

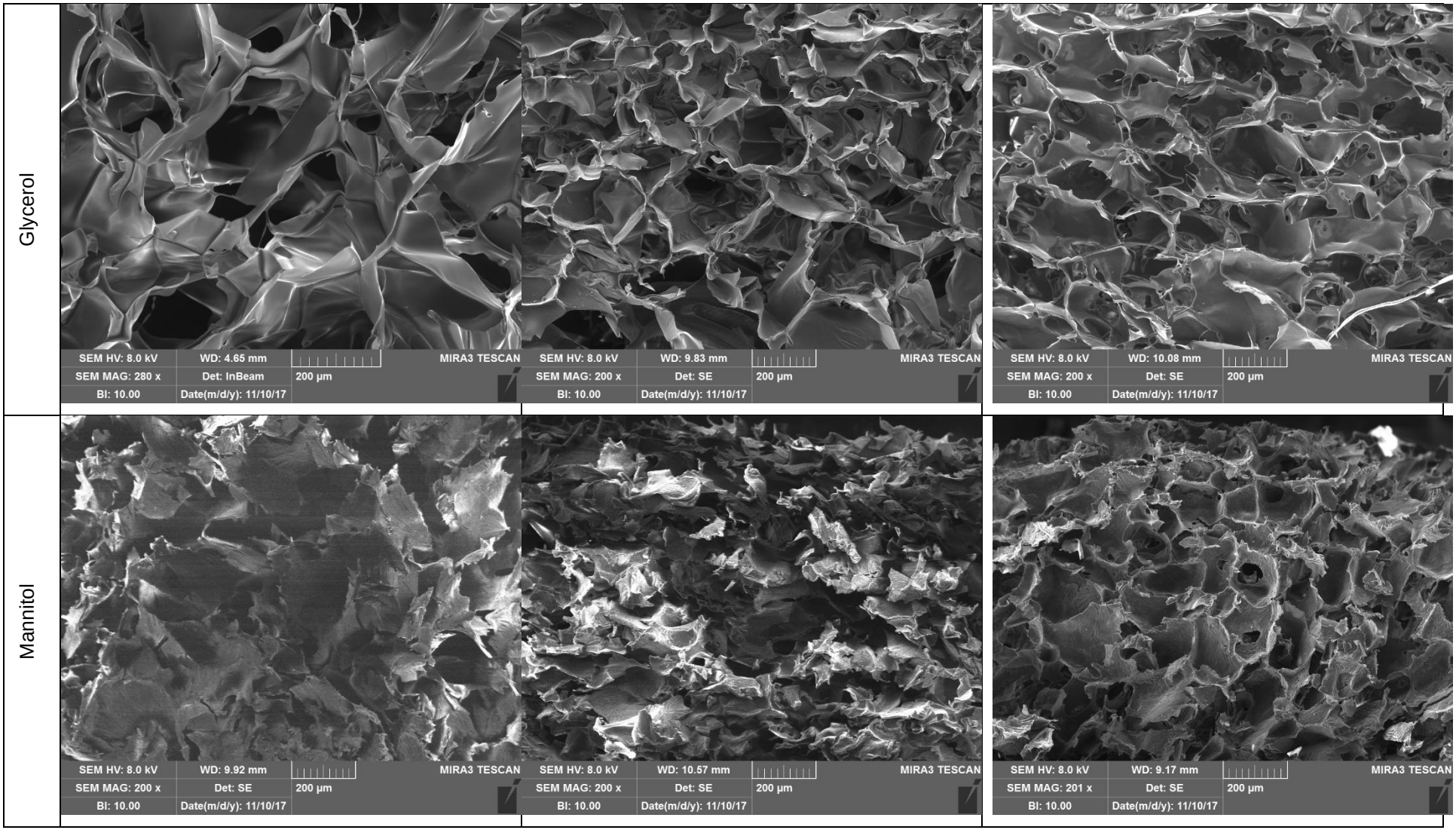


Figure 4: SEM microphotographs of transversal sections of CH 1, CH 1- HA and CH 1 – CS unloaded dressings and TA loaded dressings without lyoprotectans, dressings with glycerol and with mannitol.

3.1.4. FT-IR analysis

Figure 5 reports the FT-IR spectra of CH 1, CH 1 – HA and CH 1 – CS HA and CH+CS dressings and of CH powder (in the inset) as comparison.

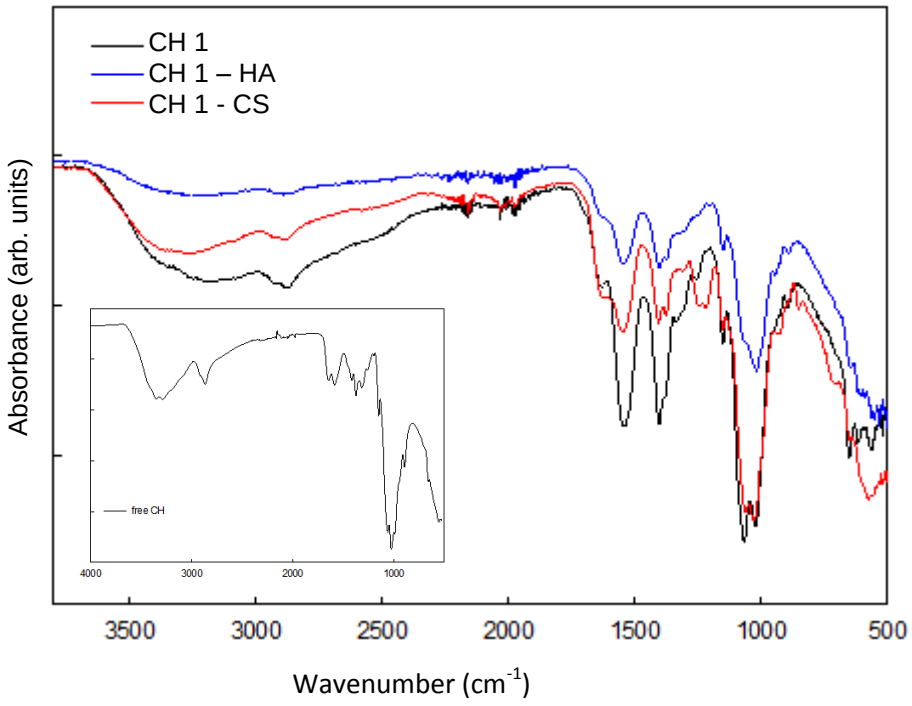


Figure 5: FT-IR spectra of CH 1, CH 1 – HA and CH 1 – CS HA and CH+CS dressings. In the inset the FTIR spectrum of CH powder (free CH) is reported as comparison.

The three spectra shared many common features both in the number and position of the peaks and seemed to be dominated by the FT-IR pattern of CH (free CH as powder), where the region around 1630 and 1540 cm^{-1} is typical of the amide I and amide II stretching and bending modes (Radhakumary, Antony, Sreenivasan, 2011; Jayakumar, Prabhakaran, Nair, Tamura, 2010). Interestingly, the position of these peaks shifted to lower frequency with respect to free CH (i.e. not interacting with acetate ions) shown Figure 1 inset. In particular, the –C=O stretching of the amide I in free CH moved from 1652 to 1634 cm^{-1} when it interacted with acetate ions and it remained in the same position even in the CH 1 - HA and CH 1 - CS systems. Moreover, a new peak around 1404 cm^{-1} (correlated to the –C-H- bending) appeared in the spectra of CH 1 dressing with respect to free CH and was found also in the spectra of CH 1 - HA and CH 1 - CS. The two mentioned spectral features have been correlated to charge-charge neutralization (salification) which most probably occurred between CH and acetate ions and dominated the spectra of all the three systems shown in Figure 1 (Shanti Krishna, Radhakumary, Sreenivasan, 2015).

This suggests that, in presence of acetate ions, the charge-charge interaction, involving chitosan amino groups, was preferentially between CH and acetate and therefore the further addition of HA and CS mostly interact with chitosan through less strong bonding such as polar interactions. Such results are in agreement with zeta potential values determined.

3.1.7. In vitro drug release properties

Figure 6 reports TA release profiles as function of time obtained for TA loaded dressings without lyoprotectans.

All the dressings were characterized by the same release behavior. TA is a water soluble drug (167 mg/ml) and considering the experimental conditions, the maximum concentration reached in the receiving phase was 1 mg/ml. In these conditions, TA release from all the dressings was relatively fast: the 50% of TA was released in about 30 min and reached quantitative and plateau value within about 3 h. This behavior should deliver TA at the application site, in the wounded bleeding lesion, to perform its procoagulant function as quickly as possible and moreover to sustain hemostatic activity for prolonged time to stabilize clot formation.

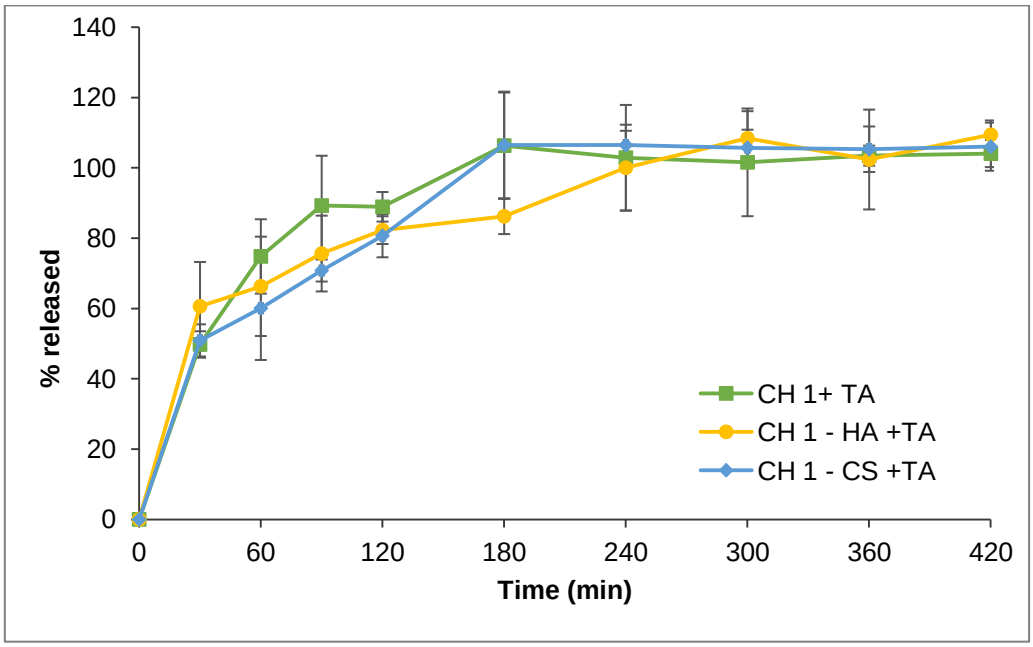


Figure 6: TA release profiles as function of time obtained for TA loaded dressings without lyoprotectans (mean values±sd; n=6).

3.2. Sponge-like dressing biopharmaceutical properties

3.2.1. In vitro dynamic whole-blood clotting

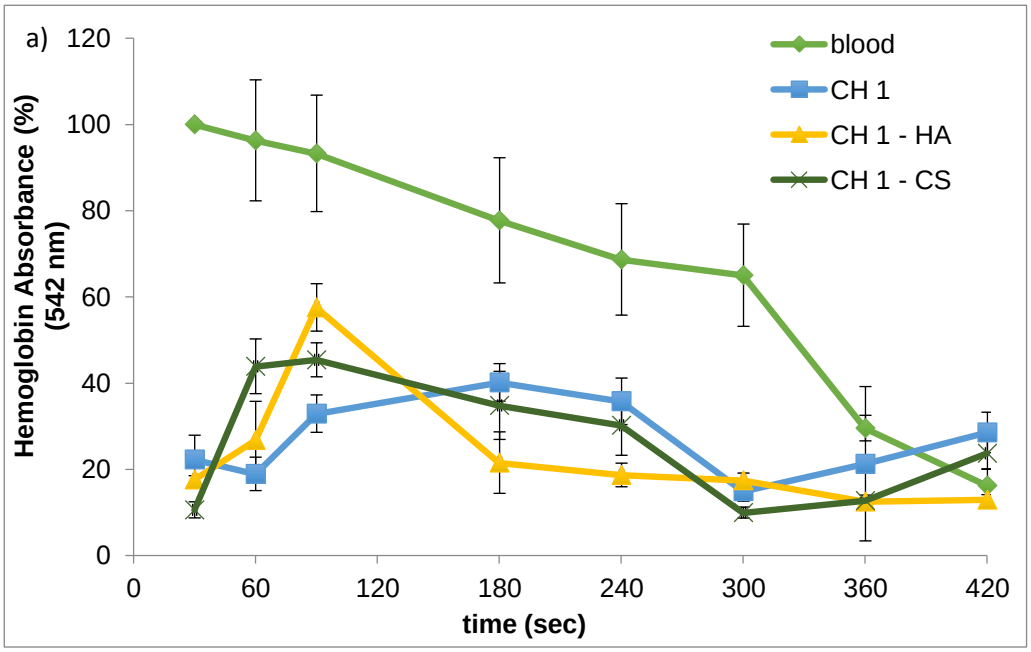
Figure 7 shows % hemoglobin absorbance profiles as function of time evaluated for a) unloaded dressings and b) TA loaded formulations.

A higher degree of hemoglobin absorbance indicates a slower clot formation rate.

Whole blood clotting formation was relatively slow and the hemoglobin absorbance reached the minimum level after about 420 s. A faster clot formation occurred in presence of all the dressings and 30 s immediately after the contact between blood and formulations the % of free hemoglobin was around 20-50%. The minimum level of hemoglobin absorbance was reached after about 300 s. Chitosan capability to bind and aggregate platelets causing agglutination of erythrocytes and activation of hemostasis was predominant in all the formulation considered also in presence of GAG.

TA is a synthetic analogue of lysine and its antifibrinolytic activity is related to reversible bindings with four to five lysine receptor sites on plasminogen or plasmin: this prevents plasmin from binding to and degrading fibrin and preserves clot structure. TA showed a synergic activity with chitosan in enhancing clot formation: all the formulations reached the minimum in hemoglobin absorbance in about 180 s, presenting a better clotting performance with respect to unloaded dressings. TA CH 1 dressing allowed to maintain a % of hemoglobin at 420 s not significantly different from initial value, while CH 1 dressing determined a significant increase in % of hemoglobin comparing the initial value with respect to the final one (at 420 s). TA CH1 –HA and TA CH1- CS dressings proved to maintain clot integrity stable from the beginning up to 420 s, while the same compositions unloaded showed a partial clot modification at 90 s with a sharp increase in % of hemoglobin.

The presence of GAG slightly decreased the procoagulant activity of the dressings. In fact, HA and CS are reported in literature as anticoagulant materials. Fragments of HA having a molecular weight lower that 500 kDa, as it is the case, cause clot structure susceptible to mechanical deformations and softening (Komorowicz, Balazs, Varga, Szabo, et al., 2016). CS is a sulfated glycosaminoglycan with heparin-like activity (Pandolfi, M., Hedner, U., 1984). On this basis, the procogulant acitivity of the systems seems exclusively due to chitosan and its synergic effect with TA.



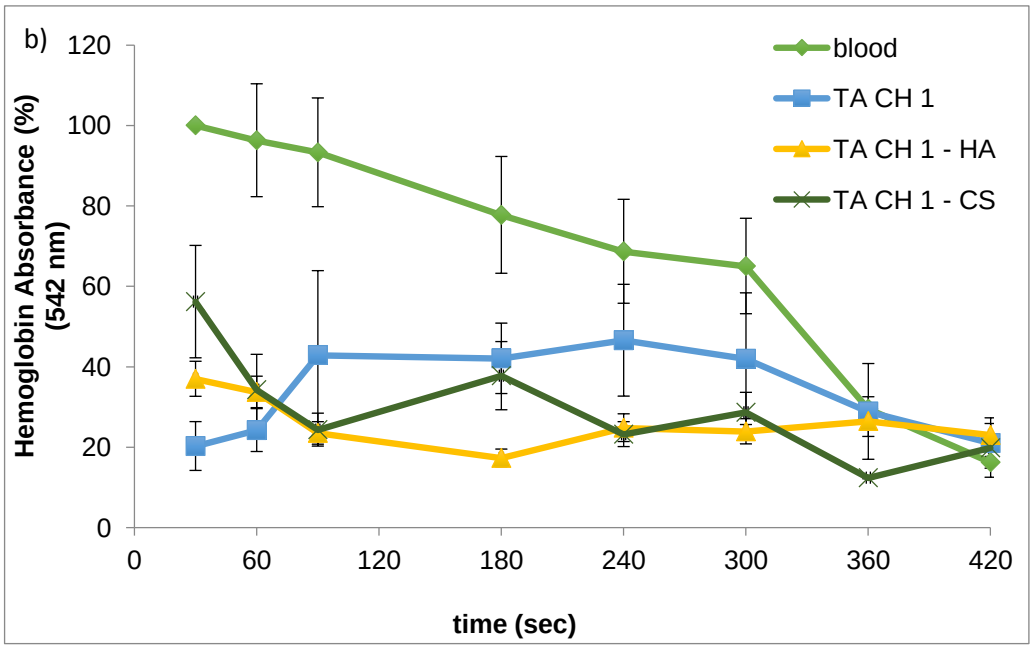


Figure 7:% hemoglobin absorbance profiles as function of time evaluated for a) unloaded dressings and b) TA loaded formulations (mean values±sd; n=6).

3.2.2. In vitro and ex vivo biocompatibility and proliferation

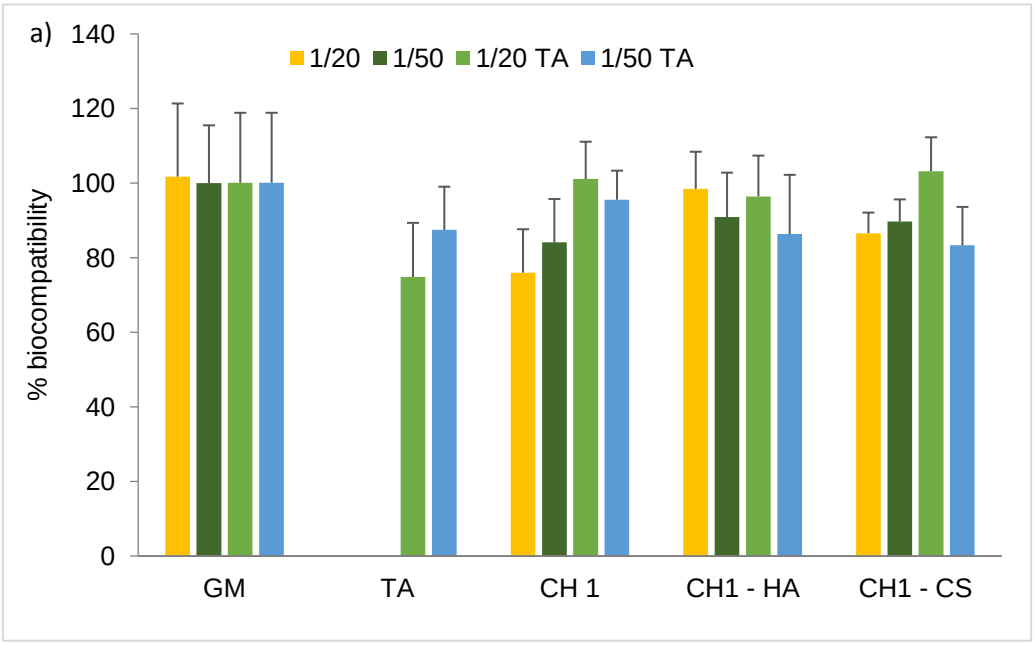
Figure 8 reports % biocompatibility (a) and % of proliferation (b) of all unloaded or TA loaded dressings (polymeric mixtures used to prepare dressings) towards fibroblasts (NHDF) for 3 and 24 h of contact time, respectively, and the amounts of proliferating nuclei/area (c) (positively stained for BrdU) counted for all the unloaded and TA loaded dressings.

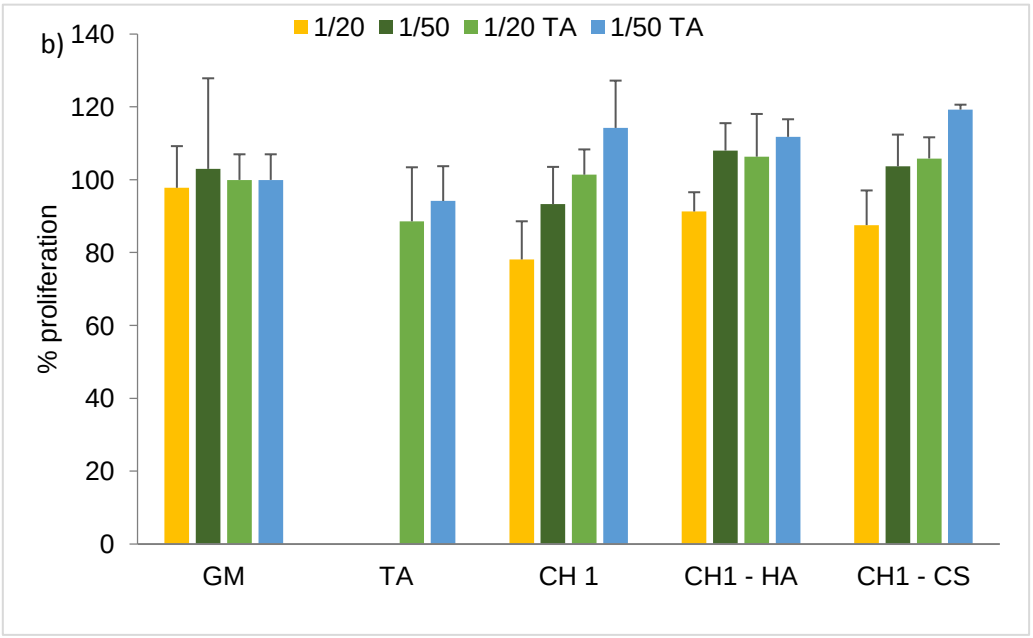
Independently of the concentrations considered, all the dressings were characterized by good biocompatibility properties towards fibroblasts also in presence of TA and the cell availability was not significantly different from those obtained with growth medium (GM, standard growth conditions).

Similarly, after 24 h the lower dressing concentrations determined the higher proliferation properties that becomes more evident in presence of tranexamic acid.

These results put in evidence that the presence of the hemostatic drug, TA, did not interfere with cell growth in the experimental conditions considered. Similar results were observed by Cholewinski et al. (2009): carotid artery derived cells maintained their morphology with toxic reaction in presence of TA.

Ex vivo skin cell proliferation (GM) was maximum in the first 24 h and decreased after 72 h and 7 days, except for CS based system that had constant activity (Figure 8 (c)). Unloaded dressings were able to sustain cell proliferation up to 7 days demonstrating a capability to enhance cell proliferation after tissue damage. TA loaded dressings were characterized by the same proliferating properties as unloaded one, considering CH 1 and CH1 - HA dressings. As for CH1 - CS dressing the presence of TA significantly increased the system proliferation properties after 24 h and 7 days and allowed to obtain the better performance, with a prolonged effect up to one week. This probably was caused by the synergic effect of TA and CS on cell growth: CS was reported as able to enhance fibroblasts and endothelial cells proliferation (Sandri, Bonferoni, Rossi, Ferrari et al., 2015) while TA could stabilize fibrin clot without impairment of cell growth and proliferation (Cholewinski, Dietrich, Flanagan, Schmitz-Rode et al., 2009). At this purpose fibrin clot was conceivably formed just after skin biopsies and it could retain growth factors released by platelets during normal physiological process, on this perspective TA, as fibrinolytic drug, could preserve the clot integrity assisting cell proliferation (Wolberg and Campbell, 2008).


a)
1/20
1/50
1/20 TA
1/50 TA
% biocompatibility
140
120
100
80
60
40
20
0
GM
TA
CH 1
CH1 - HA
CH1 - CS


b)
1/20
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1/20 TA
1/50 TA
% proliferation
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120
100
80
60
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GM
TA
CH 1
CH1 - HA
CH1 - CS

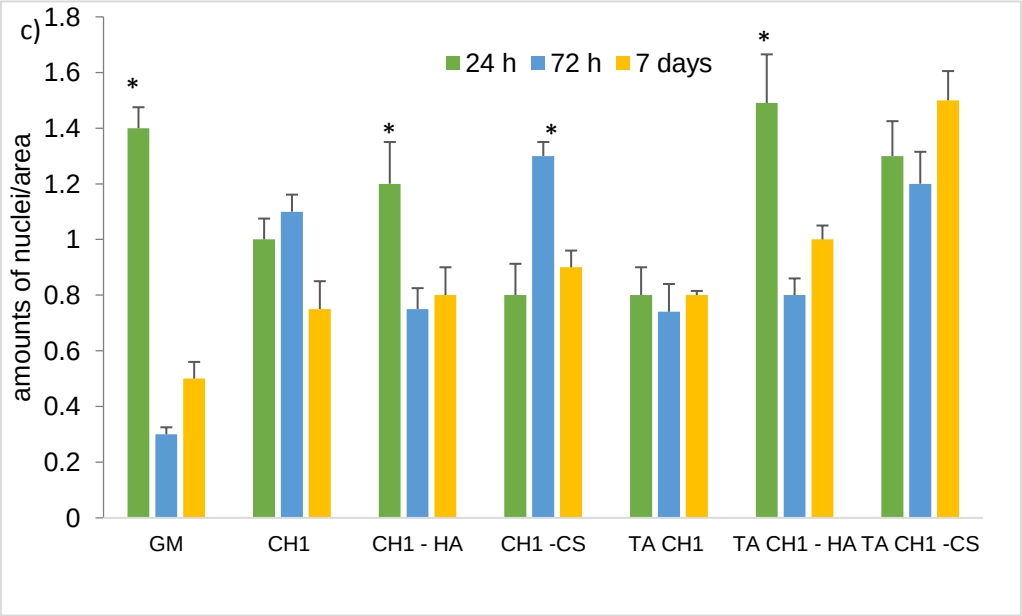

Figure 8: % biocompatibility (a) and % proliferation (b) of all unloaded or TA loaded dressings (polymeric mixtures used to prepare dressings) towards fibroblasts (NHDF) for 3 and 24 h of contact time, respectively; amounts of proliferating nuclei (c) (positively stained for BrdU) for all the unloaded and TA loaded dressings after 24 and 72 h and 7 days of contact time (mean values ± sd; n=8)

4. Conclusions

Sponge-like dressings based on chitosan and chondroitin sulfate or hyaluronic acid were prepared by lyophilization and loaded with tranexamic acid. The presence of GAG conceivably caused the occurrence of either chitosan and hyaluronic acid or chitosan and chondroitin sulfate interactions. The presence of glycosaminoglycans decreased hydration properties of the systems in a buffer simulating wound exudate – pH 7.2) and this was primarily due to dressing morphology. Chitosan based dressings had a beehive structure with polyhedric cavities of about 500 μm, interconnected by pores having oval or round shapes (50 to 200 μm) while dressings based on chitosan in association with GAG markedly decreased the cavity dimensions (200-300 μm) and with smaller pore diameters (about 50) μm. Liquid absorption is a key point in dressings to ensure hemostasis, to control wound bed hydration enhancing grunulation phase and healing. The presence of GAG in dressings significantly enhanced dressing bioadhesive properties considering as biological substrate egg-shell membrane to mimic wound bed. The association of chitosan with GAG allowed the consolidation of bioadhesive joint probably via hydrogen bonds that strengthen chitosan bioadhesion behavior, mainly due to charge-charge interaction between chitosan and the biological substrate. This is a

functional property since bioadhesion is fundamental to favor an intimate and prolonged contact between wound dressings and lesion, avoiding formulation detachment and increasing hemostatic potential.

TA release was fast to allow procoagulant function as quickly as possible and moreover to sustain hemostatic activity for prolonged time, to stabilize clot, moreover TA presented a synergic effect with chitosan to speed up clotting formation.

In vitro and ex vivo evaluations on fibroblasts and human skin, respectively, evidenced that the developed dressings enhanced cell proliferation.

Bartley (2013) suggested that the association of chitosan with TA should improve hemostatic control and should lead to improved clinical outcomes in internal surgical wounds. In this paper, sponge-like dressings based on chitosan and GAG and loaded with TA demonstrated to combine both hemostasis and proliferation properties and it seemed promising in control bleeding and healing in wounds as well as in abdominal surgery.

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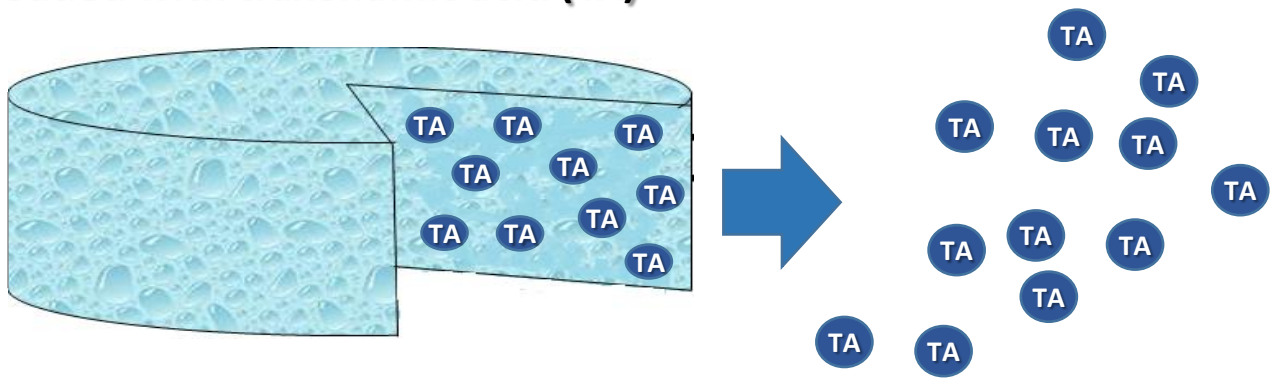
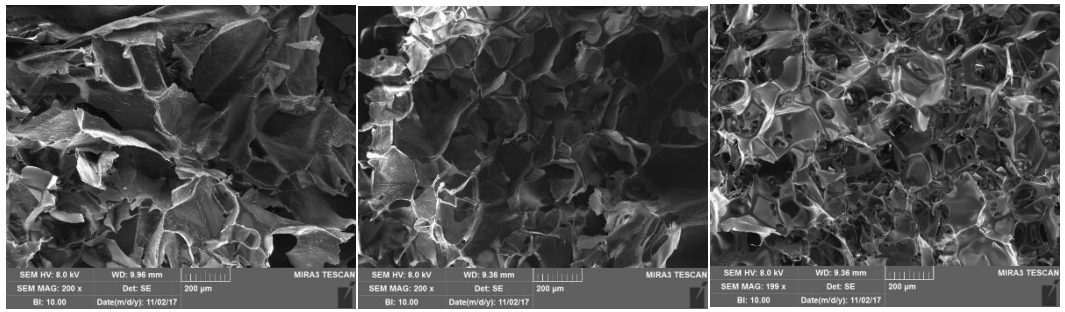
952

Graphical Abstract

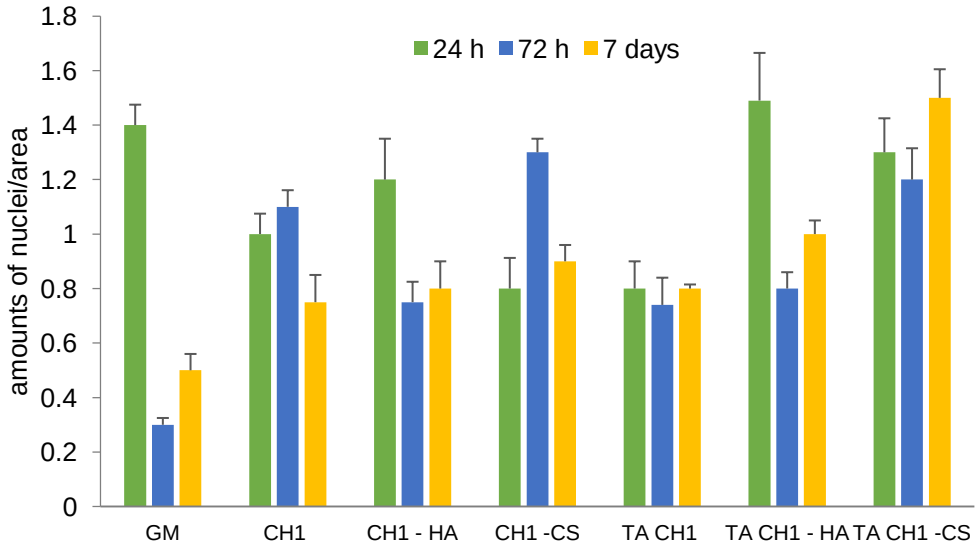
Chitosan-glycosaminoglycan based sponge like dressings loaded with traxenamic acid (TA)





chitosan | chitosan hyaluronic acid | chitosan chondroitin sulfate

ex vivo proliferation on human skin



In vitro whole blood clotting