Dodecenylsuccinic anhydride modified collagen hydrogels loaded with simvastatin as skin wound dressings.

Christian E. Olivetti[a], María I. Alvarez Echazu[a], Oriana Perna[a], Claudio J. Perez[c], Romina Mitarotonda[a,d], Mauricio De Marzi[d], Martín F. Desimone[b] and Gisela S. Alvarez[b]

[a] Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica. Junín 956, Piso 3° (1113), Buenos Aires, Argentina.

[b] Universidad de Buenos Aires, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). Instituto de Química y Metabolismo del Fármaco (IQUIMEFA), Facultad de Farmacia y Bioquímica. Junín 956, Piso 3° (1113), Buenos Aires, Argentina.

[c] Instituto de Investigaciones en Ciencia y Tecnología de Materiales (INTEMA), Grupo Ciencia y Tecnología de Polímeros. Universidad Nacional de Mar del Plata, Juan B. Justo 4302, (7600) Mar del Plata, Argentina

[d] Laboratorio de Inmunología, Instituto de Ecología y Desarrollo Sustentable (INEDES) CONICET-UNLu Departamento de Ciencias Básicas, Universidad Nacional de Luján, Ruta 5 y Avenida Constitución (6700) Luján, Buenos Aires, Argentina.

*Corresponding author e-mail: gialvarez@ffyb.uba.ar

Telephone number: 54-1152874333

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/jbm.a.36713

Abstract

Skin wound healing presents a unique challenge because of its complex healing process. Herein, we developed a hydrophobic wound dressing to incorporate simvastatin, which has potential application in the treatment of ulcers and prevention of wound infection. For that matter, collagen hydrogels were grafted with dodecenylsuccinic anhydride (DDSA). The chemical modification was confirmed by FTIR and solid state ^{13}C-NMR spectroscopies while the ultrastructure was observed by SEM images. In contact angle measurements, a higher water droplet angle in DDSA-collagen gels was observed. This was consistent with the swelling assay, in which water absorption was 5.2 g/g for collagen and 1.9 g/g for DDSA-collagen. Additionally, viability and adhesion studies were performed. Cell adhesion decreased ~11% in DDSA-collagen and the number of viable cells showed a tendency to decrease as DDSA concentration increased but it was only significantly lower above concentrations of 12%. Modified gels were loaded with simvastatin showing higher adsorption capacity and lower release. Lastly, the antimicrobial and anti-inflammatory activity of DDSA-collagen materials were assessed. DDSA-collagen hydrogels, either unloaded or loaded with simvastatin showed sustained antimicrobial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus* for 72 h probably due to the hydrophobic interaction of DDSA chains with bacterial cell walls. The antimicrobial activity was stronger against *S. aureus*. Collagen hydrogels also presented a prolonged antibacterial activity when they were loaded with simvastatin, confirming the antimicrobial

properties of statins. Finally, it was observed that these materials can stimulate resident macrophages and promote an M2 profile which is desirable in wound healing processes.

Keywords

Collagen, dodecenylsuccinic anhydride, simvastatin, wound healing, antimicrobial, M2 macrophages

1. Introduction

The drug simvastatin has been commonly used for its hypolipemiant properties acting as a 3-hidroxi-3-metil-glutaril-CoA (HMG-CoA) reductase inhibitor. However, recent studies have shown that it has a wide range of applications unrelated to cholesterol reduction including antioxidant, anti-inflammatory [(1)], and antifibrotic effects [(2)] as well as angiogenic activity [(3),(4)]. Topical application of simvastatin has shown an anti-inflammatory action in acute irritant contact dermatitis induced by croton oil in animal models and at the same time statins have been used topically for the treatment of skin disorders such as acne, seborrhea and rosacea [(5)].

The local application of simvastatin has also been reported in periodontal regenerative therapy [(6)] and in the stimulation of bone tissue regeneration [(7)] while other authors have investigated the effect of simvastatin in diabetes-related healing defects in a skin wound model indicating that simvastatin administration restored the impaired wound healing

process in diabetic mice [8]. Similar results were observed for topically administered simvastatin resulting in a significant acceleration of wound recovery due to an increase in both angiogenesis and lymphangiogenesis. [9] However, other investigators compared the effect of collagen sponges loaded with simvastatin and unloaded for the topical treatment of human ulcers and did not found statistically significant differences between the two groups regarding absolute changes in wound areas and healing areas per day, while the percentages of changes in healing rates were even better for unloaded sponges [10].

Finally, another important effect reported for statins includes its antimicrobial activity against important Gram-positive pathogens, in particular methicillin-resistant *S. aureus* and against Gram-negative pathogens as well, once the barrier imposed by the outer membrane is permeabilized. In this case, the mechanism of action proposed may involve inhibition of multiple biosynthetic pathways and cellular processes, including selective interference with bacterial protein synthesis [11]. Simvastatin was the only statin with activity against clinical isolates and reference strains of methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA). It exhibited a Minimal Inhibitory Concentration (MIC) of 15.65 µg/mL for *S. aureus* (ATCC 29213) and 31.25 µg/mL for the other strains of *S. aureus* tested. In addition, simvastatin inhibits adhesion and biofilm formation at concentrations from 1/16 x MIC to 4 x MIC and was also able to act against mature biofilms, reducing cell viability and extra-polysaccharide production [12]. Moreover, topical application of simvastatin at its MIC against *S. aureus* accelerated the healing and bacterial clearance of bacteria contaminated wounds in an excisional mice wound model [13].

On the other hand, collagen dressings have been extensively used for wound healing as they restore the barrier function lost during injury, while stimulating cell migration and supporting the growth of new cells in the affected area [14],[15]. Collagen is the most abundant structural protein of connective tissues in mammals and shows good biocompatibility and biodegradability [16],[17]. It has been obtained from different sources including rat tail, fish, bovine, ovine or porcine [18],[19]. Additionally, it has been used with the intention to deliver certain drugs or biomolecules [20],[21] that contribute to the healing process or limit and prevent microbial colonization of the wound using mainly antibiotics [22],[23] or natural products [24],[25]. However, collagen scaffolds often present poor mechanical properties, high susceptibility to degradation and low capacity to incorporate hydrophobic drugs making necessary physical and chemical modifications to improve their properties [26],[27]. Regarding collagen mechanical properties some strategies have been used to improved them including its concentration through evaporation procedures until concentrations of 40 $mg.ml^{-1}$ [28], compression, cross-linking with reticulant agents or by the association of collagen with mineral phases to produce composites [29]. With respect to the administration of drugs from collagen hydrogels many efforts have been made in order to achieve a controlled delivery as they are poor drug delivery systems releasing the drug content in short times [30]. Some of them include the preparation of collagen nanocomposites using silica nanoparticles [22][31], the formation of inclusion complexes with cyclodextrins [32] or covalent interactions between drugs and wound dressing substrates [33].

Several strategies have been investigated for the oral delivery of statins in order to increase their *in vitro* dissolution and bioavailability due to their hydrophobic nature [34] but less information can be found in the field of topical application [35] where this kind of lipophilic drugs are typically included in vehicles such as solid lipid nanoparticles[36] o liposomes [37].

For its part, dodecenylsuccinic anhydride (DDSA) is a cyclic anhydride which can react with the functional groups OH and NH_2 which are present in the chemical structure of collagen. It has been used to introduce an hydrophobic moiety in predominantly hydrophilic structures like proteins and polysaccharides in order to alter their properties in terms of dispersibility, water interaction, film forming capacity, homogeneity and to develop drug delivery systems of poorly soluble active pharmaceutical ingredients [38]. In this aspect, an amphiphilic gelatin macromolecule capable of self-assembling to form micelle-like nanospheres was developed for the entrapment of hydrophobic therapeutic molecules by grafting hydrophobic hexanoyl anhydrides to the amino groups of gelatin [39]. In the same way, hyaluronic acid was modified with octenyl succinic anhydride to obtain a local/controlled delivery platform of an hydrophobic anti-inflammatory drug [40].

In this work, type I collagen was derivatized with dodecenylsuccinic anhydride with the intention to obtain more hydrophobic wound dressings, which could potentially incorporate higher amounts of the drug simvastatin for their application in the treatment of ulcers and prevention of wound infection. In order to confirm that DDSA was covalently bound to hydroxyl or amine groups of collagen amino acids, infrared and solid NMR were used. The material obtained was further characterized by rheology measurements, electron

microscopy, enzymatic degradation with collagenase and its water uptake capability. Simvastatin incorporation in pure collagen hydrogels and DDSA- modified collagen gels was evaluated and compared together with the drug release profile from both materials. Moreover, cell adhesion and proliferation on this material was assayed, as well as its antimicrobial activity against common pathogens involved in wound infection. Finally, a monocyte cell line was exposed to the materials and pro-inflammatory and anti-inflammatory cytokines were measured to evaluate macrophage activation.

2. Materials and Methods

2.1 Reagents and Materials

(2- Dodecen- 1-yl) succinic anhydride and thiazolyl blue tetrazolium bromide reagent were purchased from Sigma–Aldrich (St Louis, USA). Dulbecco´s modified Eagle´s medium, penicillin, streptomycin and collagenase Type I (340 U/mg) were supplied by Gibco/ Life technologies. All other reagents were of analytical grade.

2.2 Synthesis of collagen hydrogels

Collagen was obtained from rat tails. Internal fibers were isolated and washed three times using buffer phosphate saline (PBS). Then, the washed fibers were placed into a 0.5 M sterile acetic acid solution, and stirred for 48 hours at 4°C. After that, the resultant colloid was centrifuged and the supernatant was collected into an empty bottle, whereas a 4 M

NaCl solution was added until the colloid reached a final concentration of 1 M NaCl and stored overnight at 4°C. Subsequently, the supernatant was discarded, and the pellet was dialyzed against a 0.5 M acetic acid solution through a 12000–14,000 molecular weight cut-off dialysis tubing for 72 h.

Finally, a 5 mg/mL collagen colloid was placed into a 24- multiwell plate, and exposed to ammonia vapor in order to neutralize the acid and form the corresponding hydrogel.

2.3 Hydrogel chemical modification with DDSA

Collagen hydrogels of approximately 5 mg obtained in the previous step were incubated with 1 mL alcoholic DDSA solutions ranging from 3% to 12% w/v under ammonia vapors to keep the pH alkaline in order to favor the reaction [41] and to prevent collagen hydrolysis caused by DDSA acid by-products. The reaction was performed a 37 °C and left for 12 hrs. Once the reaction was finished, the gels were exhaustively washed until a pH of 7.0 was reached. In parallel, the same reaction was performed with 1 mL of a collagen solution (5 mg/mL) mixed with alcoholic DDSA solutions to give a final concentration ranging from 3% to 12% w/v. The reaction conditions were the same that in the case of collagen hydrogels but the reaction took place at the same time than collagen polymerization. These gels will be called hybrids in the rest of the text.

2.4 Electron microscopy characterization

The morphology of the developed structures was observed with a Zeiss Supra 40 scanning electron microscope (SEM). Samples were washed with PBS, fixed with a 2.5 % glutaraldehyde in PBS solution for 1 h at 4ºC, freeze dried and subjected to gold sputtering prior to analysis.

2.5 Rheological measurements

The elastic or storage modulus, G'(w), and the viscous or loss modulus, G'' (w), of the materials under study were obtained in small-amplitude oscillatory shear flow experiments using a rotational rheometer from Anton Paar (MCR-301) provided with a CTD 600 thermo chamber. The tests were performed using parallel plates of 25 mm diameter, a frequency range of 0.1– 10 s^{-1}, at room temperature (22° C). All the tests were performed using small strains to ensure the linearity of the dynamic responses. All the samples were tested in triplicate using different samples. The gap width used was 1300 mm.

2.6 FT-IR spectroscopy assay and solid- state NMR characterization

FT-IR spectroscopy assay was performed on the different lyophilized hydrogel films using a Nicolet is 50 FT-IR spectrophotometer, with a KBr beamsplitter. Spectra were obtained with a resolution of 2 cm^{-1} using a DTGS detector. The samples were measured using the Attenuated total reflectance (ATR) technique.

All solid-state ^{13}C NMR experiments were performed at room temperature in a 300 MHz Bruker Avance II spectrometer equipped with a 4-mm magic angle spinning (MAS) probe.

High-resolution ^{13}C solid-state spectra were recorded using the ramp {1 H}-{13C} CP-MAS. The acquisition time was 2 ms. The spinning rate was 10 kHz for all the samples and experiments. The characterization by means of spectroscopy techniques was applied to 6% DDSA- modified hydrogels.

2.7 Collagenase assay

Resistance of 6% DDSA-modified hydrogels to enzymatic digestion was evaluated using a collagenase assay. Hydrogels with a volume of 500 μL were incubated for 24 h in 1 mL of PBS at room temperature until neutral pH was reached. Subsequently, the PBS 1X solution was substituted for 1 mL of bacterial collagenase solution (15 units/mL of collagenase type I in PBS Buffer). After incubation at 37 °C at different time points, the enzymatic reaction was halted by centrifugation, posterior discarding of the supernatant and freezing at -18°C, following vacuum dehydration. The remaining mass of the hydrogels was measured and normalized to the remaining mass of non-digested hydrogels.

2.8 Water Uptake Rate and Capacity

Pure collagen and 6% DDSA-modified hydrogels were freeze-dried. Afterwards, they were weighted (W_o) and soaked in PBS at 37°C to allow water uptake. Hydrogels were removed from PBS and weighted (W) at different time points (t) after removing the excess of water. The degree of swelling was calculated using the following equation:

$W\% = [(W - W_0)\ W_0] \times 100$

2.9 Contact angle measurements.

Contact angles were determined on dried films by averaging measurements on three distilled water droplets using a Ramé-Hart 190 contact-angle apparatus.

2.10 Cell adhesion and viability studies on scaffolds

Pure collagen and DDSA-modified hydrogels in different concentrations ranging from 1.6 to 12 % were exposed to the fibroblastic cell line L929 of mouse origin. For this purpose, 5 x 10^4 cells were added on top of each gel along with 1 mL of cell culture medium. For proliferation experiments, the medium was removed, replaced with 0.45 mL of fresh media and 0.05 mL of a 5 mg/mL MTT solution and incubated in a humidified 5% carbon dioxide chamber for 4 h. Following incubation, MTT solution was removed, gels were washed three times with PBS and 1 mL of absolute ethanol was added before leaving to stand for 30 minutes. The absorbance was recorded at 570 nm and readings were converted to cell number with a standard curve.

For adhesion studies, the cells were seeded onto prepared scaffolds at a density of 50,000 cells per gel, they were left until they could adhere for 4 h at 37°C and after the time elapsed, non-adherent cells were removed by washing with PBS. The MTT assay was performed over adhered cells.

2.11 Simvastatin incorporation and release studies

Simvastatin incorporation in collagen and 6% DDSA-modified hydrogels was analysed. In this sense, both hydrogels were incubated with different concentrations of alcoholic simvastatin solutions in air-tight tubes. After 24h, the remaining solution was employed to quantify simvastatin by an HPLC method using a 5µm C8 column of 150mm x 4.6 mm and with an UV-detector working at a wavelength of 238nm. The mobile phase was a methanol/acetonitrile/water mixture (70:10:30) adjusted to pH 2.5 with phosphoric acid and the flow rate was 1.0 ml/min [(42)].

For the release studies, collagen and DDSA collagen materials (45 mg) loaded with simvastatin were placed with 10 mL of PBS at 37 °C for 1, 2, 4, 6, 24, 48 and 72 h. The amount of drug released was measured at the different periods employing the HPLC method described before. During each collection time, the solution was completely replaced with fresh PBS. The remaining amount of simvastatin in the gels after 72 h was eluted with ethanol and also quantified.

2.12 Antimicrobial activity evaluation

Staphylococcus aureus (ATCC 29213) and *Pseudomonas aeruginosa* (ATCC 27853) were incubated overnight at 37°C in Luria-Bertani (LB) broth (yeast extract, 5 g/L; NaCl, 10 g/L and triptone, 10 g/L). A bacterial suspension of 1.10^6 CFU/mL in PBS was added to each pure collagen or 6% modified hydrogel preloaded with simvastatin solutions in concentrations of 10 and 100 µM prepared from a concentrated simvastatin solution of

1mM in dimethyl sulfoxide. The hydrogels were then incubated overnight at 37 °C with 0.2 ml of a bacterial suspension. After 24 h, the bacterial suspension on the top of the hydrogels was withdrawn and serial dilutions were made in physiologic solution. Finally, 20 µL of each dilution were spread in agar plates and the number of colony forming units was counted. This methodology was repeated two more times, to evaluate the sustained antimicrobial effect of the synthesized materials after 24 h, 48 h and 72 h.

Moreover, the minimum inhibitory concentration was determined by broth dilutions assay with *S. aureus* bacterial suspensions of 1.0 10^6 CFU/ml. Serial simvastatin dilutions were prepared from 30 to 0.5 µg/ml.

2.13 Anti-inflammatory activity of simvastatin loaded materials

The monocyte cell line THP-1 (ATCC® TIB-202™) derived from human peripheral blood from a one-year-old male was used to obtain macrophages for the essays. THP-1 cells were expanded in RPMI medium with 10% heat-inactivated fetal bovine serum (FBS), and 100 U/ml penicillin and 100 µg/ml streptomycin sulphate. Monocytes were seeded at 6 x10^5 cell/ml on collagen and DDSA-collagen gels loaded or not with simvastatin in a concentration of 100 µM and incubated for 6 h. After that, THP-1cells were differentiated to macrophages through incubation with 100ng/ml of lipopolysaccharide (LPS) for 24 h.

Nitric Oxide (NO) production was determined in the supernatant of cultures by using Griess reaction assay [(43)]. In addition, the secretion of interleukin-1β (IL-1), interleukin-6 (IL-6), interferon gamma (IFN-γ) and transforming growth factor beta (TGF-β) were measured by ELISA from the supernatant (SN) of cultures with different treatments to evaluate the M2 effect of simvastatin. Concentrations of cytokines were determined using ELISA kits according to the manufacturer's instructions (BD, OptEIATM).

2.14 Statistical analysis

All experiments were performed at least in triplicate and statistically analysed by one-way ANOVA. Data are represented as means ± SD. The differences were analysed using one-way ANOVA, followed by Bonferroni's Multiple Comparison Test, when $p < 0.05$ difference was considered significant.

3. Results

3.1 Electron microscopy characterization

DDSA-modified collagen gels were obtained in two different ways, through esterification after the gelation step or through the formation of a mixed gel (hybrid) during fibrillogenesis by exposure to ammonia vapors. SEM microscopy was used to determine if collagen fibers and hydrogel porosity were significantly altered after the reaction with DDSA and to evaluate the morphology and overall structure of hybrid materials. It was possible to observe by SEM photographs a marked difference in the morphology of both types of gels obtained by the different procedures. In the first case, the formation of typical collagen fibers with their characteristic striated appearance was conserved in DDSA-modified hydrogels under all the tested conditions. No significant differences between morphologies of the treated and common collagen scaffolds were noted. In the second case, when hybrid materials were analysed, collagen fibers were not formed and a material with

lower porosity and a honeycomb-like shape similar to that observed for gelatin scaffolds was obtained instead [44]. Therefore, it can be presumed that addition of DDSA during the gelation process hinders the self-assembly of collagen molecules into fibrils and this effect could be due to protein denaturalization because of reaction conditions like temperature and the presence of ethanol [45] (Figure 1 a-d).

3.2 Rheological measurements

Mechanical properties of the materials were studied by rheological measurements and compared to collagen hydrogels. Figure 2 displays the linear viscoelastic behavior of storage modulus (G') and loss modulus (G'') of hybrid and DDSA-collagen hydrogels in different concentrations.

For all materials, the storage modulus G' was much higher than the loss modulus G", as expected for hydrogels with significant elastic properties. The values of G' increased almost three times for DDSA- modified hydrogels in comparison to pure collagen matrices. This value slightly increased with the increase in DDSA concentration from 3 to 6% indicating that they were more stable to deformation by external forces probably because of the cross-linking of collagen molecules [46],[47] which suggests that both carboxylic groups of the anhydride and not only one were involved in the reaction. In the case of hybrid scaffolds, a 2-fold increment was observed for 3% DDSA treated materials but at higher concentrations the G' values were below those observed for pure collagen. This observation probably indicates that at 3% concentration of DDSA in hybrid materials the denaturation

of collagen due to reaction conditions may not be complete, coexisting with the cross-linking of remaining collagen fibrils.

Modified collagen gels with concentrations above 6 % DDSA were also assayed but with difficulty due to the fragility of the material. Therefore, the 6% DDSA concentration was chosen for the rest of the experiments performed.

Hybrid DDSA-collagen materials did not conserve the structure of typical collagen scaffolds as observed by SEM microscopy. Moreover, this arrangement of collagen molecules in the scaffold played a critical role on its strength and mechanical properties. It has been reported that collagen´s triple helix structure has to be preserved in order to keep its properties during the wound healing process [(48)]. For this reason, hybrid materials were discarded and no further examined for the rest of the assays.

3.3 FT-IR spectroscopy assay and solid- state NMR characterization

The signal differences between a 6% DDSA-modified gel and a pure collagen hydrogel can be observed in the IR spectrum (Figure 3), identifying new signals in the 2880 cm^{-1} region corresponding to the characteristic vibration of the C-H stretch of the carbon chain incorporated with the reaction and an increment in the signal at 1662 cm^{-1} (Amide I) which is attributed to the stretching of the C = O groups formed by the amidation reaction in the modified gels. It is also possible to observed, in both situations, the amide II signal at 1560 cm^{-1} for N-H bending vibrations coupled with C-N stretching. The amide III vibration band at~1220 cm^{-1} and the band at 1450 cm^{-1} associated to the pyrrolidine ring vibration of proline and hydroxyproline are slightly diminished in DDSA cross-linked collagen gels.

Furthermore, solid-NMR spectroscopy was used to confirm the covalent bonding of DDSA to collagen amino acids and to elucidate which of them were involved in the linking. As it can be observed in Figure 4, the signal at 160 ppm is assigned to the C_ε of the arginine residues which is present in the pure collagen spectra and hardly observed in the DDSA-modified gel. In the same way, the peak assigned to the C_4 Hyp at 70 ppm and C_5 Lys at 30 ppm are clearly affected after treatment with DDSA [49],[50]. These carbons are located in a position adjacent or near a hydroxyl (Hyp) or amine group (Arg and Lys) where the DDSA might have reacted to form a covalent union altering or shifting the signal observed in the NMR spectra. The peaks around 15 ppm can be attributed to the methyl carbons of the hydro carbonated tail of DDSA incorporated to the structure.

It is also possible to evidence a marked sharpening of some signals, especially those at 175 ppm which correspond to amide carbon signals, esters and carboxylate carbons.

Considering that in solid NMR a cause of signal broadening is molecular disorder and environmental heterogeneity, the signal sharpening observed could be attributed to the covalent bonding of DDSA residues which create a more ordered polymer [51].

3.4 Collagenase assay

The susceptibility of collagen hydrogels to accelerated digestion by collagenases at 37°C can be seen in Figure 5. Both pure and DDSA-collagen gels were incubated with collagenase solutions for a period of 24 h. DDSA-modified gels presented a more limited degradation, losing only 40 percent of their initial weight after 3 h and remaining in 50% after 24h while pure collagen gels lost 80% of their weight after 3h and were completely degraded after 24h. This effect could be attributed to the blockage of functional groups by DDSA that may disfavor enzyme recognition and interaction with its substrate.

3.5 Water Uptake Rate and Capacity

Collagen hydrogels can absorb a small amount of fluids by swelling in the wound, but they can donate moisture to a dry wound and maintain a moist wound environment which is favorable for the healing process. In the case of collagen sponges or films, they are not useful to absorb exudates in wounds with heavy drainage like polyurethane foams, for instance, but they can be applied to wounds with low to moderate exudates [52].

The water uptake of these materials was also evaluated as demonstrated in Figure 6. The swelling capacity of pure collagen was double than that of chemically modified materials indicating the hydrophobic nature of the synthetized dressings. The incorporation of water was 5.2 g water/g dried scaffold in the case of collagen and 1.9 g water/g dried for DDSA-modified collagen scaffolds. Even though the incorporation of water capacity was reduced after the modification of collagen, these values were still in the same range than commercial hydrocolloid products used as wound dressings like DuoDERM, Hydro Coll or Tegaderm which were reported to incorporate water in the range of 0.9 for HydroColl to 1.9 g water/g dried for Tegaderm [(53)].

3.6 Contact angle measurements

Water contact angle was used to quantify the surface wettability of the scaffolds (Figure 7) giving values of 18.6° for pure collagen and 35.3° for DDSA-modified gels. As it is natural to expect, the surface of collagen films showed a rather low contact angle of water which indicates their hydrophilicity due to the presence of a greater content of acidic, basic, and hydroxylated amino acid residues rather than lipophilic ones. In the case of DDSA-modified films, the increment in the contact angle observed is a good evidence of the chemical modification proposed and the incorporation of hydrophobic domains in the collagen structure, although the material still conserved an overall hydrophilic nature with low contact angle values.

3.7 Cell adhesion and viability studies on scaffolds

Collagen hydrogels were grafted with different concentrations of DDSA ranging from 1.6 to 12 %. Then a fibroblastic cell line was seeded on top of the different scaffolds obtained and finally subjected to a viability assay for 72 h period. As it can be observed in Figure 8a, the number of viable cells showed a tendency to decrease as the reagent concentration increased but it was only significantly lower (*c.a.* 40%) when the DDSA concentration employed was higher than 12%.

Therefore, in the second study (Figure 8b), the DDSA concentration was fixed in 6% to evaluate if the lower number of cells detected in modified-gels was due to an initial inferior cell adhesion or to limited proliferation of fibroblasts in the scaffolds. The cell adhesion to the modified gels was slightly lower, around 11%, than that of pure collagen gels. The proliferation studies after 72 h showed similar results to those observed in the first assay, with approximately 27% less viable cells in 6 % DDSA-modified gels. These results suggest that the increment in superficial hydrophobicity due to the chemical modification on collagen scaffolds can affect cell adhesion as expected by previous studies [(54),(55)] and also DDSA incorporation may altered cell viability. However, it is still possible for cells to colonize, grow and spread in this new material.

3.8 Simvastatin incorporation and release studies

Drug incorporation into collagen and DDSA-collagen gels was evaluated to determine if the chemical modification could be useful for the delivery of hydrophobic therapeutic agents (Figure 9A).

The results are shown as the amount of adsorbed drug (in μg/g dried gel) expressed as a function of equilibrium concentration in the solution (in μmol/L). It was seen that these isotherms fitted the Freundlich adsorption model which reflects the surface heterogeneity of the material, leading to different adsorption forces and different affinities toward drug molecules. The values of the constants were K=0.09553, N= 0.9473 for collagen gels and K=0.2498, N=0.8818 for DDSA-collagen gels with R^2 of 0.9685 and 0.9800, respectively. Although the two gels could adsorb simvastatin, DDSA-collagen materials possess a higher adsorption capacity due to a superior K value.

Based on the data found in literature about the effect of simvastatin on cell viability it was decided to pre-incubate collagen scaffolds with simvastatin concentrations of 10 μM and 100 μM. Simvastatin is commonly used in cell culture in concentrations ranging from 50 to 0.01 μM as higher amounts of this drug can affect cell viability [56],[57].

In this situation, it was observed that simvastatin incorporation after incubating with 10 μM solutions was approximately 0.7 μg/g dried gel and 1.5 μg/g for collagen and DDSA-collagen matrices. When the incubation was performed with 100 μM solutions, the amounts obtained were 6.1 μg/g and 16.9 μg/g, respectively.

Simvastatin release profiles were obtained for both types of hydrogels, collagen and DDSA-collagen, and they are presented in Figure 9B. As it is possible to observe after 6 h,

near 33 and 37 % of the drug was released from collagen and DDSA-collagen dressings, respectively. Following that initial burst release, a constant value was registered for the two matrices in the 72 h period of the assay indicating a controlled release with similar kinetics for the two materials. At this time point, 51% of the drug was released from collagen while 45 % was registered for DDSA-collagen gels due to the higher affinity of the hydrophobic drug for the hydro carbonated tails introduce to the proteinaceous polymer.

.

3.9 Antimicrobial activity evaluation

Antibacterial properties were analysed using *Pseudomonas aeruginosa* and *Staphylococcus aureus* ATCC strains, comparing the activities between the DDSA- modified hydrogels and non-modified hydrogels unloaded or loaded with either 10 μM or 100 μM simvastatin solutions. As shown in Figure 10, in the case of *S. aureus*, pure collagen hydrogels presented a different behavior when they were loaded or not with simvastatin. In this case, a significant difference was obtained for both concentrations tested (10 and 100 μM) reducing around 1 or 2 logarithmic orders of magnitude. On the third day, only gels containing higher amounts of simvastatin (100 μM) exhibited antimicrobial effects. This confirms the antimicrobial effects of simvastatin reported by other authors [(12),(13)] and it is supported by the determination of the minimum inhibitory concentration (MIC) calculated against *S. aureus* which was 5.6 μg/ml. Furthermore, these results are in agreement with the prolonged release of simvastatin observed for over 3 days from collagen gels.

The antimicrobial effect of DDSA-modified gels was superior to that observed for pure collagen hydrogels both for unloaded and simvastatin loaded materials but there was no significant difference when DDSA gels were carrying the drug simvastatin or not. It has been reported that DDSA-gum Karaya in concentrations from 1 to 10% and DDSA-gum Kondagogu presented antibacterial effects for both gram negative and gram positive bacteria [(58)]. In the same way, DDSA-chitosan gels exhibited greater growth inhibition of Gram-positive bacteria as compared to Gram-negative by the hydrophobic interaction of dodecenyl succinyl chains with bacterial cell wall proteins [(59)]. Therefore, DDSA could be masking simvastatin antimicrobial properties as they probably do not behave in a synergistic way. On the third day, even though not significantly different, a higher antibacterial effect can be observed for simvastatin loaded gels in comparison to unloaded DDSA-modified gels.

On the other hand, for *P. aeruginosa* exposed to pure collagen gels loaded with simvastatin, it was only possible to observe a growth inhibition effect during the first day with a decrease of more than 6 log units. In agreement with previous authors, Gram negative bacteria are less susceptible to simvastatin antimicrobial effect and need higher concentrations to be affected. DDSA-modified gels had a similar effect during the first day, in the unloaded or loaded condition. During the third day, it was also possible to detect a significantly different antibacterial activity for 100 μM simvastatin carrying DDSA-collagen gels.

3.10 Collagen materials loaded with simvastatin modulate anti-inflammatory activity of macrophages

Pro and anti-inflammatory cytokine production as well as nitric oxide (NO) levels were measured in undifferentiated and differentiated THP-1 cells, named as THP-1 monocytes and THP-1 macrophages (after LPS stimulation) when they were in contact with collagen and DDSA-collagen hydrogels loaded or not with simvastatin. As it can be observed in Figure 11 A and B, NO levels were higher when monocytes were exposed to DDSA-collagen gels loaded with simvastatin but after stimulation with LPS no significant difference was found in the different conditions. This is in concordance with literature where statins effect on the upregulation of nitric oxide synthase was described [(60)].

Considering that cells produce NO in response to various pathogens and that it is a molecule implicated in host defense, no increment in NO levels was observed after LPS stimulation of cells. The levels of pro-inflammatory cytokine IL-6 were significantly lower in the case of collagen gels loaded with simvastatin and DDSA-collagen gels either with or without simvastatin (Figure 11 C). In the case of IL-1 β no differences were observed with respect to the control (Figure 11 D). Furthermore, the presence of IFN-γ in the cell medium was measured (data not shown) and the levels were below the detection limit of the technique. LPS normally causes a significant increase in the levels of IL-6, IL-1ß and TNF-γ (M1 phenotype) which was not observed for collagen hydrogels. In the case of TGF-ß1 (Figure 11 E) which levels also increase after exposure to LPS, the amounts detected were lower than the control, especially in the case of DDSA-collagen gels loaded

with simvastatin. M1 macrophages are differentially expressed after the administration of LPS which produce pro-inflammatory cytokines, however, the administration of collagen gels loaded with simvastatin might be able to polarize this response preferentially to M2 macrophages.

4. Discussion

Collagen hydrogels were successfully modified by treatment with DDSA in alkaline conditions leading to a new material which preserved its fibrillar and porous structure with enhanced mechanical properties, better resistance to enzymatic degradation, increased hydrophobicity and antibacterial and anti-inflammatory activities. The chemical modification was corroborated by means of IR spectroscopy and solid state-NMR where it was possible to predict that DDSA interaction sites were mainly amine groups of lysine and arginine residues and the hydroxyl group of hydroxyproline. The increment in the scaffold hydrophobicity was observed by contact angle measurements and was responsible for the higher incorporation of simvastatin, a hydrophobic drug, inside DDSA-modified gels. These evidence together with the slightly slower release profiles suggest that DDSA-collagen gels could be used as delivery systems for water insoluble drugs.

The higher mechanical resistance observed after DDSA modification together with the slower enzymatic degradation are clear advantages for the design of wound dressings that can resist the attack of physiological enzymes present in the patient and last longer without

substitution [61]. Although cell attachment and proliferation in the scaffolds were diminished after DDSA-modification, they still exhibited good cytocompatibility and the possibility of cell spreading. Cytotoxicity can be rated based on cell viability relative to controls, where values of <30% are considered severe cytotoxicity, between 30 and 60% is moderate cytotoxicity, between 60 and 90% is slight cytotoxicity, and >90% is no cytotoxicity [62]. For DDSA-collagen materials the viability observed was above 70% with respect to collagen gels which is considered a slightly cytotoxic material that can be used for biomedical applications.

Additionally, DDSA-collagen gels showed antibacterial properties mainly against Gram positive bacteria due to the increased hydrophobicity which is believed to be favorable in preventing water and bacteria intrusion for collagen-based biomaterials [63]. It has also been demonstrated that pathogenic bacteria and fungi have hydrophobic characteristics that make them irreversibly bind strongly to hydrophobic wound dressings by a physical principle [64]. Moreover, when bacteria come into contact with the hydrocarbonated tails exposed in the gel, death may occur as a consequence of wall disruption. It has also been reported that bacterial cellulose wound dressings carrying long-chain unsaturated fatty acids are promising antimicrobial agents of natural origin with a broad spectrum of activity [65].

On the other hand, collagen hydrogels loaded with simvastatin were also effective in reducing the number of bacteria in contaminated media for as long as 3 days. It has been proved that topical application of statins may be useful in the wound healing process not only because of their antibacterial effect but also due to their angiogenic, antioxidant and

anti-inflammatory properties. In this sense, it has been observed that especially DDSA-collagen gels carrying simvastatin can modulate an M2 profile for THP-1 cells which are involved in anti-inflammation and tissue repair.

The physical and chemical properties of biomaterials can alter or modulate the response by macrophages. For example, it has been reported that calcium and strontium ions on a nanostructure titanium surface can increase M2 macrophage phenotype [(66)] or hydroxyapatite granules activate some M1, but more M2 activation of THP-1 cells [(67)]. Another study reported that carbonated hydroxyapatite in the form of a coating rather than in granules was a potent M1 stimulator for THP-1 cells, and then transitioned to M2 via simvastatin delivery [(68)]. In the present studies, we demonstrated that DDSA-collagen gels and materials carrying simvastatin were modulators of an M2 profile for THP-1 cells. Moreover, the presence of simvastatin in the biomaterial reduces pro-inflammatory cytokines that drive the M1 phenotype. Others authors have previously shown that anti-inflammatory actions of simvastatin result in better repair of the wounds [(69)].

The purpose of collagen wound dressings would not be their application in an infected wound. In fact, they are not recommended in those cases [(52)]. However, they are useful in maintaining a moist environment for the wound, facilitating autolytic debridement and promoting granulation and epithelialization. Furthermore, when they are used as drug delivery vehicles they can prevent wound contamination. In this situation, it is advisable to polarize the macrophages towards the M2 phenotype that contributes to reduce wound inflammation and promotes wound healing [(70)].

Although, M2 phenotype would not contribute to the elimination of bacteria, it is important in the regeneration of the wound. In this sense, one of the main problems of wounds, besides the settlement of bacterial pathogens, is the exacerbated immune response in the site of the injury. The administration of simvastatin, with its pleiotropic effects, not only prevents the establishment of infections, but also displays anti-inflammatory properties that prevent a major tissue damage.

Conclusion

Collagen hydrogels used as wound dressings have shown to be permeable to gas and water and have proven to be a less effective bacterial barrier than occlusive dressings [(52)]. For this reason, strategies to decrease bacterial contamination of the wound and to promote wound healing are desired. In conclusion, both materials, DDSA-collagen gels and collagen hydrogels loaded with simvastatin showed antimicrobial and anti-inflammatory properties which make them promising for cutaneous wound healing. DDSA-collagen hydrogels also exhibited better mechanical properties and lower degradation rates and the possibility to function as drug carriers for water insoluble drugs.

Acknowledgements

C.E Olivetti, M. I. Alvarez Echazu and O. Perna are grateful for their doctoral or student fellowships granted by Universidad de Buenos Aires. The authors would also like to

acknowledge the support of grants from the Universidad de Buenos Aires UBACYT 20020130300043BA and 20020150100056BA, from CONICET PIP 11220120100657CO and from Agencia Nacional de Investigaciones Científicas y Técnicas PICT-2013-2570 (to G.S.A) and PICT-2015-2922 (to M. F. D).

Conflicts of interest

The authors report no conflict of interest.

References

1. Jain MK, Ridker PM. Anti-Inflammatory Effects of Statins: Clinical Evidence and Basic Mechanisms. Nat Rev Drug Discov. 2005;4:977–87.
2. Moriyama T, Kawada N, Nagatoya K, Takeji M, Horio M, Ando A, Imai E, Hori M. Fluvastatin suppresses oxidative stress and fibrosis in the interstitium of mouse kidneys with unilateral ureteral obstruction. Kidney Int. 2001;59:2095–103.
3. Katsumoto M, Shingu T, Kuwashima R, Nakata A, Nomura S, Chayama K. Biphasic effect of HMG-CoA reductase inhibitor, pitavastatin, on vascular endothelial cells and angiogenesis. Circ J. 2005;69:1547–55.
4. Liao JK, Laufs U. Pleiotropic effects of statins. Annu Rev Pharmacol Toxicol. 2005;45:89–118.
5. Jowkar F, Namazi MR. Statins in dermatology. Int J Dermatol. 2010;49:1235–43.
6. Elavarasu S, Suthanthiran TK, Naveen D. Statins: A new era in local drug delivery. J

Pharm Bioallied Sci. 2012;4:S248-51.

7. Yan Q, Xiao LQ, Tan L, Sun W, Wu T, Chen LW, Mei Y, Shi B. Controlled release of simvastatin-loaded thermo-sensitive PLGA-PEG-PLGA hydrogel for bone tissue regeneration: In vitro and in vivo characteristics. J Biomed Mater Res - Part A. 2015;103:3580–9.
8. Bitto A, Minutoli L, Altavilla D, Polito F, Fiumara T, Marini H, Galeano M, Calò M, Cascio P Lo, Bonaiuto M, Migliorato A, Caputi AP, Squadrito F. Simvastatin enhances VEGF production and ameliorates impaired wound healing in experimental diabetes. Pharmacol Res. 2008;57:159–69.
9. Asai J, Takenaka H, Hirakawa S, Sakabe JI, Hagura A, Kishimoto S, Maruyama K, Kajiya K, Kinoshita S, Tokura Y, Katoh N. Topical simvastatin accelerates wound healing in diabetes by enhancing angiogenesis and lymphangiogenesis. Am J Pathol. 2012;181:2217–24.
10. Raposio E, Libondi G, Bertozzi N, Grignaffini E, Grieco MP. Effects of Topic Simvastatin for the Treatment of Chronic Vascular Cutaneous Ulcers: A Pilot Study. J Am Coll Clin Wound Spec. 2015;7:13–8.
11. Thangamani S, Mohammad H, Abushahba MFN, Hamed MI, Sobreira TJP, Hedrick VE, Paul LN, Seleem MN. Exploring simvastatin, an antihyperlipidemic drug, as a potential topical antibacterial agent. Sci Rep. 2015;5:16407.
12. Graziano TS, Cuzzullin MC, Franco GC, Schwartz-Filho HO, De Andrade ED, Groppo FC, Cogo-Müller K. Statins and antimicrobial effects: Simvastatin as a

potential drug against Staphylococcus aureus biofilm. PLoS One. 2015;10.

13. Wang CC, Yang PW, Yang SF, Hsieh KP, Tseng SP, Lin YC. Topical simvastatin promotes healing of Staphylococcus aureus-contaminated cutaneous wounds. Int Wound J. 2015;13:1150–7.

14. Udhayakumar S, Shankar KG, Sowndarya S, Rose C. Novel fibrous collagen-based cream accelerates fibroblast growth for wound healing applications: in vitro and in vivo evaluation. Biomater Sci. 2017;5:1868–83.

15. Lee CH, Singla A, Lee Y. Biomedical applications of collagen. Int J Pharm. 2001;221:1–22.

16. Echazú MIA, Tuttolomondo MV, Foglia ML, Mebert AM, Alvarez GS, Desimone MF. Advances in collagen, chitosan and silica biomaterials for oral tissue regeneration: from basics to clinical trials. J Mater Chem B. 2016;4:6913–29.

17. Hsu S, Hung K-C, Chen C-W. Biodegradable polymer scaffolds. J Mater Chem B. 2016;4:7493–505.

18. Ghodbane SA, Dunn MG. Physical and mechanical properties of cross-linked type I collagen scaffolds derived from bovine, porcine, and ovine tendons. J Biomed Mater Res - Part A. 2016;104:2685–92.

19. Pal P, Srivas PK, Dadhich P, Das B, Maity PP, Moulik D, Dhara S. Accelerating full thickness wound healing using collagen sponge of mrigal fish (Cirrhinus cirrhosus) scale origin. Int J Biol Macromol. 2016;93:1507–18.

20. Li X, Wang J, Su G, Zhou Z, Shi J, Liu L, Guan M, Zhang Q. Spatiotemporal control

over growth factor delivery from collagen-based membrane. J Biomed Mater Res A. 2011;100:396–405.

21. Wang X, Thibaud C, Helary C. Modulating inflammation in a cutaneous chronic wound model by IL-10 released from collagen–silica nanocomposites via gene delivery. Biomater Sci. 2018;6:398–406.

22. Alvarez GS, Hélary C, Mebert AM, Wang X, Coradin T, Desimone MF. Antibiotic-loaded silica nanoparticle–collagen composite hydrogels with prolonged antimicrobial activity for wound infection prevention. J Mater Chem B. 2014;2:4660.

23. Hall Barrientos IJ, Paladino E, Szabó P, Brozio S, Hall PJ, Oseghale CI, Passarelli MK, Moug SJ, Black RA, Wilson CG, Zelkó R, Lamprou DA. Electrospun collagen-based nanofibres: A sustainable material for improved antibiotic utilisation in tissue engineering applications. Int J Pharm. 2017;531:67–79.

24. Kandhasamy S, Perumal S, Madhan B, Umamaheswari N, Banday JA, Perumal PT, Santhanakrishnan VP. Synthesis and Fabrication of Collagen-Coated Ostholamide Electrospun Nanofiber Scaffold for Wound Healing. ACS Appl Mater Interfaces. 2017;9:8556–68.

25. Govindarajan D, Duraipandy N, Srivatsan KV, Lakra R, Korapatti PS, Jayavel R, Kiran MS. Fabrication of Hybrid Collagen Aerogels Reinforced with Wheat Grass Bioactives as Instructive Scaffolds for Collagen Turnover and Angiogenesis for Wound Healing Applications. ACS Appl Mater Interfaces. 2017;9:16939–50.

26. Perumal RK, Perumal S, Thangam R, Gopinath A, Ramadass SK, Madhan B, Sivasubramanian S. Collagen-fucoidan blend film with the potential to induce fibroblast proliferation for regenerative applications. Int J Biol Macromol. 2018;106:1032–40.

27. Ward J, Kelly J, Wang W, Zeugolis DI, Pandit A. Amine functionalization of collagen matrices with multifunctional polyethylene glycol systems. Biomacromolecules. 2010;11:3093–101.

28. Helary C, Bataille I, Abed A, Illoul C, Anglo A, Louedec L, Letourneur D, Meddahi-Pellé A, Giraud-Guille MM. Concentrated collagen hydrogels as dermal substitutes. Biomaterials. 2010;31:481–90.

29. Giraud Guille MM, Nassif N, Fernandes FM. Collagen-based Materials for Tissue Repair, from Bio-inspired to Biomimetic. Mater Des Inspired by Nat Funct Through Inn Archit. 2013;4:107–27.

30. Wallace DG, Rosenblatt J. Collagen gel systems for sustained delivery and tissue engineering. Adv Drug Deliv Rev. 2003;55:1631–49.

31. Mebert AM, Aimé C, Alvarez GS, Shi Y, Flor SA, Lucangioli SE, Desimone MF, Coradin T. Silica core–shell particles for the dual delivery of gentamicin and rifamycin antibiotics. J Mater Chem B. 2016;4:3135–44.

32. Chen Y, Song W, Zhao X, Han Q, Ren L. An antibacterial collagen membrane crosslinked by the inclusion complex of β-cyclodextrin dialdehyde and ofloxacin for bacterial keratitis. RSC Adv. 2018;8:18153–62.

33. Hu S, Cai X, Qu X, Yu B, Yan C, Yang J, Li F, Zheng Y, Shi X. Preparation of biocompatible wound dressings with long-term antimicrobial activity through covalent bonding of antibiotic agents to natural polymers. Int J Biol Macromol. 2019;123:1320–30.

34. Tiwari R, Pathak K. Nanostructured lipid carrier versus solid lipid nanoparticles of simvastatin: Comparative analysis of characteristics, pharmacokinetics and tissue uptake. Int J Pharm. 2011;415:232–43.

35. Tanigo T, Takaoka R, Tabata Y. Sustained release of water-insoluble simvastatin from biodegradable hydrogel augments bone regeneration. J Control Release. 2010;143:201–6.

36. Saporito F, Sandri G, Bonferoni MC, Rossi S, Boselli C, Cornaglia AI, Mannucci B, Grisoli P, Vigani B, Ferrari F. Essential oil-loaded lipid nanoparticles for wound healing. Int J Nanomedicine. 2018;13:175—186.

37. Cheng R, Yan Y, Liu H, Chen H, Pan G, Deng L, Cui W. Mechanically enhanced lipo-hydrogel with controlled release of multi-type drugs for bone regeneration. Appl Mater Today. 2018;12:294–308.

38. Shah NN, Soni N, Singhal RS. Modification of proteins and polysaccharides using dodecenyl succinic anhydride: Synthesis, properties and applications—A review. Int J Biol Macromol. 2018;107:2224–33.

39. Li W-M, Liu D-M, Chen S-Y. Amphiphilically-modified gelatin nanoparticles: Self-assembly behavior, controlled biodegradability, and rapid cellular uptake for

intracellular drug delivery. J Mater Chem. 2011;21:12381.

40. Mayol L, Biondi M, Russo L, Malle BM, Schwach-Abdellaoui K, Borzacchiello A. Amphiphilic hyaluronic acid derivatives toward the design of micelles for the sustained delivery of hydrophobic drugs. Carbohydr Polym. 2014;102:110–6.

41. Wang H, Williams PA, Senan C. Synthesis, characterization and emulsification properties of dodecenyl succinic anhydride derivatives of gum Arabic. Food Hydrocoll. 2014;37:143–8.

42. Sultana N, Arayne MS, Shah SN, Shafi N, Naveed S. Simultaneous determination of prazosin, atorvastatin, rosuvastatin and simvastatin in API, dosage formulations and human serum by RP-HPLC. J Chinese Chem Soc. 2010;57:1286–92.

43. Griess P. Bemerkungen zu der abhandlung der H.H. Weselsky und Benedikt “Ueber einige azoverbindungen.” Chem Ber. 1879;12:426–8.

44. Johari B, Ahmadzadehzarajabad M, Azami M, Kazemi M, Soleimani M, Kargozar S, Hajighasemlou S, Farajollahi MM, Samadikuchaksaraei A. Repair of rat critical size calvarial defect using osteoblast-like and umbilical vein endothelial cells seeded in gelatin/hydroxyapatite scaffolds. J Biomed Mater Res - Part A. 2016;104:1770–8.

45. Gopinath A, Reddy SMM, Madhan B, Shanmguam G, Rao JR. Effect of aqueous ethanol on the triple helical structure of collagen. Eur Biophys J. 2014;43:643–52.

46. Tian Z, Li C, Duan L, Li G. Physicochemical properties of collagen solutions cross-linked by glutaraldehyde. Connect Tissue Res. 2014;55:239–47.

47. Foglia ML, Camporotondi DE, Alvarez GS, Heinemann S, Hanke T, Perez CJ, Diaz

LE, Desimone MF. A new method for the preparation of biocompatible silica coated-collagen hydrogels. J Mater Chem B. 2013;1:6283.

48. Fleck CA, Simman R. Modern collagen wound dressings: Function and purpose. J Am Col Certif Wound Spec. 2010;2:50–4.

49. Aliev AE. Solid-state NMR studies of collagen-based parchments and gelatin. Biopolymers. 2005;77:230–45.

50. Saitô H, Yokoi M. A13C NMR study on collagens in the solid state: Hydration/ dehydration-induced conformational change of collagen and detection of internal motions. J Biochem. 1992;111:376–82.

51. Romer FH, Underwood AP, Senekal ND, Bonnet SL, Duer MJ, Reid DG, Van Der Westhuizen JH. Tannin fingerprinting in vegetable tanned leather by solid state NMR spectroscopy and comparison with leathers tanned by other processes. Molecules. 2011;16:1240–52.

52. Sood A, Granick MS, Tomaselli NL. Wound Dressings and Comparative Effectiveness Data. Adv Wound Care. 2014;3:511–29.

53. Wang HM, Chou YT, Wen ZH, Wang ZR, Chen CH, Ho ML. Novel Biodegradable Porous Scaffold Applied to Skin Regeneration. PLoS One. 2013;8.

54. Webb K, Hlady V, Tresco PA. Relative importance of surface wettability and charged functional groups on NIH 3T3 fibroblast attachment, spreading, and cytoskeletal organization. J Biomed Mater Res. 1998;41:422–30.

55. Lotfi M, Nejib M, Naceur M. Cell Adhesion to Biomaterials: Concept of

Biocompatibility. Adv Biomater Sci Biomed Appl. 2013.

56. Yazawa H, Zimmermann B, Asami Y, Bernimoulin J-P. Simvastatin Promotes Cell Metabolism, Proliferation, and Osteoblastic Differentiation in Human Periodontal Ligament Cells. J Periodontol. 2005;76:295–302.

57. Cáceres M, Romero A, Copaja M, Díaz-Araya G, Martínez J, Smith PC. Simvastatin alters fibroblastic cell responses involved in tissue repair. J Periodontal Res. 2011;46:456–63.

58. Padil VVT, Senan C, Černík M. Dodecenylsuccinic Anhydride Derivatives of Gum Karaya (Sterculia urens): Preparation, Characterization, and Their Antibacterial Properties. J Agric Food Chem. 2015;63:3757–65.

59. Inta O, Yoksan R, Limtrakul J. Hydrophobically modified chitosan: A bio-based material for antimicrobial active film. Mater Sci Eng C. 2014;42:569–77.

60. Ma FX, Han ZC. Statins, nitric oxide and neovascularization. Cardiovasc Drug Rev. 2005;23:281–92.

61. Lee JE, Park JC, Hwang YS, Kim JK, Kim JG, Sub H. Characterization of UV-irradiated dense/porous collagen membranes: morphology, enzymatic degradation, and mechanical properties. Yonsei Med J. 2001;42:172–9.

62. Lönnroth EC. Toxicity of medical glove materials: A pilot study. Int J Occup Saf Ergon. 2005;11:131–9.

63. Xu Z, Chang J, Zhang P, Guan X, Chen Y, Fan H. Collagen modified with epoxidized safrole for improving antibacterial activity. RSC Adv. 2017;7:50300–6.

64. Ljungh A, Yanagisawa N, Wadström T. Using the principle of hydrophobic interaction to bind and remove wound bacteria. J Wound Care. 2006;15:175–80.

65. Żywicka A, Fijałkowski K, Junka AF, Grzesiak J EFM. Modification of Bacterial Cellulose with Quaternary Ammonium Compounds Based on Fatty Acids and Amino Acids and the Effect on Antimicrobial Activity. Biomacromolecules. 2018;ASAP.

66. Lee C-H, Kim Y-J, Jang J-H, Park J-W. Modulating macrophage polarization with divalent cations in nanostructured titanium implant surfaces. Nanotechnology. 2016;27:085-101.

67. Fernandes KR, Zhang Y, Magri AMP, Renno ACM, Van Den Beucken JJJP. Biomaterial Property Effects on Platelets and Macrophages: An in Vitro Study. ACS Biomater Sci Eng. 2017;3:3318–3327.

68. Alhamdi JR, Peng T, Al-Naggar IM, Hawley KL, Spiller KL, Kuhn LT. Controlled M1-to-M2 transition of aged macrophages by calcium phosphate coatings. Biomaterials. 2018;In press:1–10.

69. Khoshneviszadeh M, Ashkani-Esfahani S, Namazi MR, Noorafshan A, Geramizadeh B, Miri R. Topical simvastatin enhances tissue regeneration in full-thickness skin wounds in rat models. Iran J Pharm Res IJPR. 2014;13:263–269.

70. Zhiyuan F, Junjie D, Peter L, Daphney C, Yunfei S, Pu Z, Kambayashi T, Elizabeth B, Lin H, Cheng H. A new class of biological materials: Cell membrane-derived hydrogel scaffold. Biomaterials. 2019;197:244–54.

Figure captions

Graphical abstract. DDSA modification of collagen hydrogel to achieve an increment in wound dressing hydrophobicity for the delivery of non-water soluble drugs with antimicrobial and anti-inflammatory activity.

Figure 1

SEM image of DDSA-collagen hydrogels obtained with DDSA concentrations of a) 3% and c) 12 % and hybrid scaffolds with a final DDSA concentration of b) 3% and d) 12 %.

Figure 2

Viscoelastic response of collagen, DDSA-collagen and hybrid hydrogels against frequency (G´ and G´´). G´represented in full symbols and G´´ in open symbols. -♦- Collagen hydrogel -▲-3% hybrid hydrogel -●- 6% hybrid hydrogel -★- 3% DDSA-collagen hydrogel -■- 6% DDSA-collagen hydrogel.

Figure 3

Comparison of ATR-FTIR spectra for 6% collagen-DDSA hydrogel (A) and a collagen hydrogel (B).

Figure 4

Comparison of solid ^{13}C NMR of A.) Collagen hydrogel and B.) DDSA-collagen gel

Figure 5

Comparison of enzymatic degradation using collagenase type I of a collagen hydrogel (-●-) and a DDSA-collagen gel (-▲-).

Figure 6

Degree of swelling of collagen (-●-) and DDSA-collagen (-▲-) hydrogels over a period of 24h in PBS.

Figure 7

Optical images used for contact angle measurements. A.) Collagen hydrogel B.) DDSA-collagen gel

Figure 8

A.)[3T3] Mouse fibroblast cytocompatibility in collagen hydrogels and DDSA-modified gels with DDSA concentrations in the range of 1.6 to 12 %. B.) Fibroblast adhesion after 4 h and viability after 72h in collagen (blue) and 6% DDSA-collagen (green) gels in

comparison to a control (100%). * $p<0.05$ was considered significant using one-way ANOVA, followed by Bonferroni multiple comparisons test or student t test.

Figure 9

A.) Freundlich adsorption isotherms for simvastatin incorporation on collagen (-●-) and DDSA-collagen (-▲-) gels. B.) In vitro cumulative percent of drug release versus time profile for collagen (-●-) and DDSA-collagen (-▲-) gels.

Figure 10

Antimicrobial activity against A.) *Staphylococcus aureus* and B.) *Pseudomonas aeruginosa* of collagen and DDSA-collagen hydrogels unloaded or loaded with 10 and 100 µM simvastatin.

The differences were analyzed using one-way ANOVA, followed by Bonferroni multiple comparisons test, $p < 0.05$ was considered significant.

Figure 11

A) Nitric oxide production of THP-1 cells exposed to collagen gels (Col), simvastatin loaded collagen gels (Col-Simv), DDSA-collagen (Col-DDSA) and DDSA-collagen loaded with simvastatin (Col-DDSA-Simv). B) Nitric oxide production after stimulation with LPS. C-E) Cytokine secretion of THP-1 cells exposed to collagen gels after stimulation with LPS.