FORMULATION OF AN ANTIMICROBIAL TOPICAL TREATMENT BASED ON MAGNETITE-BUFORIN-II NANOBIOCONJUGATES

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Abstract

Community acquired infections caused by Methicillin Resistant Staphylococcus aureus (S. aureus) (MRSA) have become a growing concern due to its impact on the world public health. The lack of highly effective antibiotics and treatments has led to the search of novel therapies using antimicrobial agents such as antimicrobial peptides (AMPs). In order to obtain a viable administration route to counteract superficial skin infections, a topical formulation based on Magnetite-BUF-II-Ag nanobioconjugates was designed by their dispersion in O/W concentrated emulsions. The prepared topical characterization indicated that O/W emulsions were stable in time, the droplets size remained within the appropriate values (~1 µm) and their rheological properties, such as pseudoplastic and shear-thinning behavior remained unchanged for three months. Additionally, hemolysis and platelet activation tests were acceptable (i.e., 14.72 ± 2.62% and 8.06 ± 2.90%, respectively) in light of the ISO-10993 standard. Furthermore, the treatment reduced completely the growth of wild type S. aureus and Escherichia coli by the contact diffusion method. These results are important in the context of proposing new alternatives that allow treating effectively the threat posed by the antibiotic resistant bacterial strains, which jeopardize the lives of thousands of people every year.

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Introduction

Despite that bacteria are ubiquitous microorganisms, and just a small percentage of them cause infections, there is a growing concern due to their large impact on public health at a global scale [1]–[3]. This impact is related to the fact that there is an increasing level of antibiotic resistant bacteria and in this case, therapeutic options available are quite limited. Specifically, the threat posed by Staphylococcus aureus Methicillin Resistant (MRSA) infection taking into account its role in nosocomial infections and also in community-acquired infections [4], [5]. This Gram-positive bacteria is the major cause of skin and soft tissue infections [6]–[9]. The severity of the infection in
the skin is classified according to the presence of impetigo (minor infection) and eczema, ulcers, as well as lacerations (secondary infection). The infected wounds are mainly treated with topical antibiotics such as mupirocin and bacitracin (with fusidic acid as active component) [6]. However, recent reports describe an increasing number of resistance cases to these two topical treatments that have resulted in fatal losses [10]. This highlights the pressing need for new and more effective therapies for the treatment of wounds infected with MRSA. Moreover, MRSA has been considered a priority in intrahospital environments for the search of more potent antimicrobials [11]–[13].

A potential alternative to traditional antibiotics present in topical treatments are antimicrobial peptides (AMPs). These naturally occurring antimicrobial molecules are widely distributed in all kingdoms of nature going from bacteria to humans. AMPs are mainly cationic, amphipathic and, among others, exhibit diverse biological activities such as antibacterial [14], antiviral [15], antifungi [15], cytotoxic [16], wound healing [17] and immunomodulatory [18]. An attractive AMP due to its unique properties is Buforin II (BUF-II), which is a frog-derived AMP of 21 amino acids (TRSSRAGLQFPVGRVHRLLRK). BUF-II was first isolated from the stomach of *Bufo bufo gargarizans* [19], and more recently from the skin of *Sphaenorhynchus lacteus* [20], [21]. BUF-II has cationic charge (6+), an alpha helix secondary structure, and the ability to penetrate cell membranes. This is in contrast with the majority of AMPs, whose main mechanism of action is based on the disruption of cell membrane integrity [22], [23]. In this regard, BUF-II stands out for its unique and quite effective antimicrobial mechanism that involves membrane bypassing to interact with DNA and ultimately interrupt bacterial replication [21], [22]. Despite the potential of BUF-II, a major drawback is its low *in vitro* and *in vivo* stability and lifespan, which limits the possibility of considering it for commercial applications [24].

These issues have been addressed by a number of strategies including functionalization with polyethylene glycol (PEG) [24], entrapment in mesoporous materials [25], encapsulation within polymeric capsules [26], liposomes [27], and immobilization on nanomaterials [28]. In this regard, a number of studies have demonstrated that by interfacing AMPs with nanomaterials, such as silica, gold, silver and carbon nanoparticles, their stability and lifespan is considerably increased [29]–[31]. This success has been mainly attributed to unique properties such as high surface to volume ratio, size, chemical reactivity, and biological mobility and stability [28], [32], [33]. Recent successful examples include immobilization of AMP esculentin-1a on gold nanoparticles [34], Andersonin-Y1 peptide on silver nanoparticles [35] and indolicin AMP on carbon nanotubes [36].

Recent reports also indicate an increased interest in the development of smarter nanomaterials with the capability of responding to environmental changes such as pH, temperature, presence of metabolites [27], [37] or even to external stimuli such as electrical or magnetic fields [31], [38]. In this regard, one of the most attractive nanoplatforms for immobilization is magnetite nanoparticles (MNPs), which exhibit strong magnetic responsiveness that has proven useful to guide their fate and transport, and to facilitate localized thermal energy release and pharmacological targeting [32]. This has been crucial to overcome one of the major challenges of modern pharmacology, which is to assure that therapies effectively reach the site of action and maintain a prolonged biological activity therein. MNPs have also shown high biocompatibility and antimicrobial abilities on their own [28], [38]. Moreover, they have been also widely employed to immobilize other biological therapeutic molecules where they demonstrated a role in improving their thermal and long-term stability as well as their
reusability [39], [40]. Examples include the immobilization of the epidermal growth factor [41], albumin [42], and bacitracin [43].

We recently developed antimicrobial nanobioconjugates of BUF-II immobilized on MNPs as a strategy to extend its stability, efficacy, and lifespan [40]. To maintain the antimicrobial activity of BUF-II, we modified the MNPs with a polyetheramine (PEA) surface spacer prior to immobilization. The spacer maintained the peptide distant from the surface and imparted sufficient flexibility to avoid structural changes that are detrimental to its antimicrobial functionality. This approach appears suitable to address some of the major pitfalls of AMPs before their translation into clinical applications such as degradation (e.g., proteolytic, hydrolysis and oxidation) and instability.

Recent reports suggest that perhaps the most viable administration route for dermal delivery of AMPs is by a topical formulation. This is particularly useful in the case of infected wounds because it can provide localized delivery along with higher peptide concentrations at the site of action [18]. Additional advantages of this approach include avoidance of systemic toxicity and side effects, the decreased induction of bacterial resistance and low costs [44], [45]. The topical treatments can be manufactured from various materials including natural and synthetic polymers and various types of emulsified systems [46], [47]. The selection of the material is primarily defined by the possible interactions with the embedded bioactive components. In this regard, most topical treatments are based on emulsified systems mainly because they permit to combine a variety of components with different physical and chemical properties, they are effective at delivering small dosages of active agents, and the final physical and textural properties of the product can be controlled according to the product purpose or the consumer preference [48], [49]. Traditionally, emulsions have been prepared by dispersion of oil in water (O/W), (known as direct) or alternatively by dispersion of water in oil (W/O), (known as inverse) [48]. Independent of the type of emulsion, dispersion of one phase in the other is generally achieved with the aid of high speed mixing, sonication, or heat [50]. Once droplets of one phase are dispersed into the other, their natural tendency is to coalesce in time. For this reason, surface tension agents, thickeners and other excipients need to be incorporated prior to forming the dispersion [50]–[52]. The type and ratio of surfactants is determined experimentally, but there are some empirical rules of thumb such as the surfactant concentration (3% - 5% wt.), the thickener concentration (0.1% - 1% wt.), and the emollients concentration (1% - 5% wt.) [48]. Moreover, some indexes such as flow and consistency have been defined to help formulators achieve the required rheological and stability properties for a particular application [53], [54].

This work is therefore dedicated to the formulation and characterization of a novel antimicrobial topical treatment for the potential control of antibiotic resistant bacteria such as MRSA. Accordingly, we prepared O/W emulsions with dispersed Magnetite-BUF-II nanobioconjugates. We characterized the obtained topicals in terms of stability, rheological response and particle size. Additionally, we determined their antibacterial capacity against S. aureus and E. coli as well as their biocompatibility in vitro. Finally, we conducted a scaling-up experiment to assure reproducibility of the topical production at the bench scale.
Materials and Methods

Materials

Iron (III) chloride hexahydrate (97%), tetramethylammonium hydroxide (TMAH) (25%), (3-aminopropyl) triethoxysilane (APTES) (98%), N-hydroxy succinimide (NHS) (98%), N-[3-dimethylamino]-propyl]-N’-ethyl carbodiimide hydrochloride (EDC) (98%), glutaraldehyde (25%), mineral oil (>99.0%), Tween 20 (Polysorbate 20) (GC grade), Tween 80 (Polysorbate 80) (GC grade), Span 80 (Sorbitan Monooleate) (GC grade), Thrombin, Triton X-100 (Laboratory grade), sodium alginate (Pharmaceutical grade) and MTT Formazan were purchased from Sigma-Aldrich (Milwaukee, WI). Dimethyl sulfoxide (DMSO) (99.0%) was obtained from Sigma-Aldrich (Allentown, PA) and silver nitrite (AgNO₃) (99%), NH₂-PEG₈-propionic acid (98%), carbopol (poly(acrylic-acid)) (>99.0%) and Cytotoxicity Detection Kit (LDH) from Sigma-Aldrich (St. Louis, MO). Iron (II) chloride tetrahydrate (98%), dimethylformamide (DMF) (99.8%), sodium chloride (NaCl) (99.9%), sodium hydroxide (NaOH) (98%) and triethanolamine (>99.0%) was obtained from PanReac AppliChem (Spain). Dulbecco's modified eagle’s medium (DMEM), fetal bovine serum (FBS) and trypsin-EDTA (1X in PBS) were purchased from Biowest (Nuaillé, FR). Penicillin/Streptomycin (P/S) was obtained from Lonza. Mueller Hinton agar and lysogeny broth (LB) for microbiological test were from Merck-Millipore (Darmstadt, GER) and Merck-Millipore (Milwaukee, WI), respectively. LIVE/DEAD™ BacLight™ Bacterial Viability Kit L13152 were purchased from Thermo Fisher Scientific (Eugene, OR) Buforin II (BUF-II) was synthesized by GL Biochem Shanghai (Shanghai, China) and the Peptide Synthesis Facility at Pompeu Fabra University (Barcelona, Spain). Bacteria strains were S. aureus (ATCC 23235) and E. coli (ATCC 25922). Cytotoxicity was conducted in HaCaT (ATCC® CRL-2404) cells.

Synthesis and Characterization of Magnetite-PEG8-BUF-II-Ag Nanobioconjugates

A mixture of 1 g of FeCl₂·4H₂O and 2.71 g of FeCl₃·6H₂O was dissolved separately in 5 mL of distilled water to obtain 1 and 2 M solutions, respectively. Chloride solutions were then mixed and heated up until a homogeneous solution was obtained. When the solution reached 90°C, 2 mL of 2% (v/v) solution of tetramethylammonium hydroxide (TMAH) was added. At the same time, 1.6 g of NaOH were dissolved in 5 mL of Type I water (8 M solution), and this solution was also heated to 90°C. The hot NaOH solution was finally dropped at 200 µL/min into the chloride mixture under vigorous stirring at 1500 RPM. The reaction was carried out for 1 h at 90°C under continuous stirring at 1500 RPM. The obtained MNPs were washed at least 3 times with distilled water with the aid of a strong permanent magnet, and sonicated after each wash (2800 ultrasonic cleaner, Branson, MO, USA) for 15 min at 40 kHz frequency.

For surface functionalization, 100 mg of magnetite were dissolved in distilled water and sonicated vigorously until complete homogenization. In total, 2 mL of 2% (v/v) solution of TMAH, 50 µL of 99% glacial acetic acid and 100 µL of 10% (v/v) APTES were added to resuspend the MNPs. The sample was then kept for 1 h at 60 °C and 250 RPM to carry out the chemical reaction. Finally, the sample was washed several times with 1.5% (w/v) NaCl saline solution and Type I water to remove excess reagents. The separation process in between washes was aided by a strong neodymium permanent magnet.
In order to modify the surface of the nanoparticles and confer flexibility to the immobilized molecules, PEG-8 polymer was conjugated on the silanized nanoparticles obtained previously. 100 mL of silanized nanoparticles diluted in 30mL of type II water with 1mL of 2% (v/v) glutaraldehyde solution were left to react for 30 mins. Next, the polymer NH$_2$-PEG8-propionic Acid solution was added and stirred overnight, followed by several washes with type II water. A mixture of EDC/NHS, pre-dissolved in DMF, and 500 µL of BUF-II solution at 1 mg/mL in sterile NaPB were added to 100 mg of PEG8-coated magnetite nanoparticles suspended in 30mL of distilled water, previously sonicated. After 5 mins of sonication and then, 24 hours of reaction, the obtained Mag-PEG8-BUF-II nanobioconjugates were thoroughly washed with type I water with the aid of a strong magnet to remove reagents excess.

To boost the antimicrobial effect of the Mag-PEG8-BUF-II nanobioconjugates, see Fig. S1, a silver core shell was synthesized on the surface of the nanobioconjugates using silver nitrite (AgNO$_3$) as precursor. Briefly, 100 mL of 1 mg/mL nanobioconjugates solution in type I water were properly sonicated. AgNO$_3$ was then added into the solution to reach a final concentration of 10$^{-3}$ M. Next, the solution was sonicated in an ultrasonic bath (2800 ultrasonic cleaner, Branson, MO, USA) for 5 min and then magnetically stirred at 600 RPM. 75 mL of honey were then incorporated into the solution followed by magnetic stirring for 5 more minutes. pH was rapidly elevated to 8 with NaOH (5 M) under agitation at 600 RPM for 1 h at room temperature. The Mag-PEG8-BUF-II-Ag nanobioconjugates were precipitated with the aid of a strong magnet and thoroughly washed with type I water. Then, the nanobioconjugates were centrifuged at 4000 RPM for 5 min to remove the reagents excess and, finally, resuspended in type I water, sonicated to avoid aggregation, and stored at 4°C until further use.

**O/W Emulsions Preparation**

O/W emulsions were prepared with two different concentrations of dispersed phase (60% wt. and 70% wt.) by varying the hydrophilic-lipophilic balance (HLB) value (11.79 and 14). Table 1 shows the components and corresponding amounts of a typical O/W formulation. For benchmarking purposes, we included a commercially available antimicrobial topical treatment, which contains fusidic acid as the active antimicrobial component. Triethanolamine and Tween 80 were added to distilled water, whereas Span 80 was dissolved in mineral oil. Each phase was then filtered with cellulose (0.22 µm pore size, GE Healthcare, UK) and PTFE (0.45 µm pore size, FOXX Life Sciences, US) filters, respectively. The solid thickener, Carbopol, was placed over a glass petri dish and then exposed to germicidal UV light for 15 min.

An aqueous suspension of magnetite-PEG8-BUF-II-Ag nanobioconjugates (1 mg/mL) was formed by vigorous vortex agitation followed by 5 minutes of agitation in a shaker incubator. The aqueous phase of the emulsion was stirred at 250 RPM while adding the nanobioconjugate suspension dropwise. Carbopol was then added slowly to the aqueous phase under vigorous agitation. Next, the oil phase was incorporated into the aqueous phase with the aid of a peristaltic pump G1000 (Fisherbrand™, Madrid, ES) operating at 20% of its maximum velocity. The mixture was maintained under vigorous stirring at 800 RPM with an overhead stirrer Hei-TORQUE Precision 400 (Heidolph, Schwaback, GE) equipped with a 60° pitch-blade impeller until the oil phase addition was completed. To obtain a stable emulsion, the mixture was then agitated at 1200 RPM for 10 more min at room
temperature. The obtained emulsions were bottled in glass flasks and stored at room temperature until further use.

**Table 1.** List of reagents and composition of the O/W emulsion at two different concentrations of the dispersed phase and two different HLB values: 60% wt. and 70% wt. and HLB 11.79 and 14.

<table>
<thead>
<tr>
<th>Composition of O/W emulsions (%)</th>
<th>60% wt</th>
<th>70% wt</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reagents</strong></td>
<td>HLB 11.79</td>
<td>HLB 14</td>
</tr>
<tr>
<td>Water</td>
<td>36.34</td>
<td>35.54</td>
</tr>
<tr>
<td>Triethanolamine</td>
<td>0.43</td>
<td>0.43</td>
</tr>
<tr>
<td>Tween 80</td>
<td>2.80</td>
<td>3.60</td>
</tr>
<tr>
<td>Carbopol</td>
<td>0.43</td>
<td>0.43</td>
</tr>
<tr>
<td>Mineral oil</td>
<td>58.80</td>
<td>59.60</td>
</tr>
<tr>
<td>Span 80</td>
<td>1.20</td>
<td>0.40</td>
</tr>
</tbody>
</table>

**Emulsion Stability Measurements**

O/W emulsions were visually inspected for the first three days after preparation every 24 hours to evaluate for phase separation, destabilization phenomena or any other change in appearance. After this period, emulsions were stored for 90 days in a stability chamber (RGX—250E, THOMSON) at 40°C and 75% humidity. Additional to visual analysis, we monitored the Turbiscan Stability Index (TSI) daily with cumulative scans in a Turbiscan instrument (FORMULACTION SAS, L’Union, FR), which provides information regarding the destabilization phenomena of a given sample [55]. Furthermore, the same instrument allowed us to obtain information on the transmittance and backscattering percentage as additional indicators of possible destabilization. For the measurements, samples were transferred to a glass vial at room temperature while avoiding bubble formation at the top of the container. The collected scans were plotted together for comparison.

**Particle Size Analysis of Droplets in Emulsions**

Mastersizer 3000 equipment (Malvern Instruments Ltd, Worcestershire, UK) was used to determine particle size average and particle size distribution of droplets in the emulsions [56]. This was accomplished by collecting 5 measurements per sample and the averaging the obtained data. In direct emulsions, the selected dispersant was water with a refractive index of 1.33 and the material was mineral oil. Obscuration lower and upper limits were fixed at 10 to 20% for O/W emulsions.
Rheological Characterization of Emulsions

Rheology characterization of the emulsions was conducted with a DHR1 rheometer (Discovery Hybrid Rheometer, Ta Instruments, DE, US). Flow and oscillatory sweep experiments were performed to determine the rheological behavior of the emulsions and to assess changes in the storage ($G'$) and loss ($G''$) moduli. All experiments were performed with a parallel plate geometry (20 mm) and at 20°C. Flow sweep was performed between 0.1 and 200 s$^{-1}$ [57]. Additionally, to obtain information about thixotropy, the flow sweep was followed by a second flow sweep analysis between 200 and 0.1 s$^{-1}$. Data obtained from the analysis of stress vs. shear rate was fitted to the potential law model (Eq. 1) [48]:

$$\tau = m\dot{\gamma}^n, \quad \text{Equation 1}$$

Where $\tau$ is the stress, $\dot{\gamma}$ is the shear rate, $m$ is the consistency index, and $n$ is the flow index.

The oscillatory test in frequency was performed at 1% strain for frequencies between 0.01-1 Hz.

Scaling-up Process of the Emulsion at Bench Scale

Taking into account the potential of the emulsion with the dispersed nanobioconjugates to treat the nosocomial infections, the production of the emulsion was further carried out at the bench scale to evaluate the process reproducibility in the production of 3000 g of the direct emulsion. Accordingly, it was necessary to consider some scale-up parameters based on the initial assembly to assure the same synthesis operation conditions (Table S1). The followed geometrical ratios are shown in Equation S1. The impellers were designed according to the required dimensions in Autodesk® Inventor® and then 3D printed in a Ultimaker 2+ 3D printer printer (Fig. S2).

For the mixing and homogenization process, the tip velocity was kept constant during the scaling-up, the Equation 2 presents the tip velocity calculation:

$$V_t = \pi DN, \quad \text{Equation 2}$$

Where $V_t$ is the tip velocity, $D$ is the impeller diameter and $N$ is the velocity fixed in RPM.

Antimicrobial Assays

Antibacterial activity was assayed by direct contact as a modification of the disk susceptibility test described by the standard M01-A11 [58]. Briefly, bacterial cells from $S. aureus$ (ATCC 23235) and $E. coli$ (ATCC 25922) were cultured and aliquoted to reach $1 \times 10^7$ CFU. 500 µL of each treatment were dispensed, in sterile conditions, in 2 mL eppendorfs by triplicate. Subsequently, 500 µL of the $1 \times 10^7$ CFU bacteria suspension were exposed to the treatments for 2 h at 37°C. The treatments tested in this experiment were the emulsion containing 400 µg/mL of the Mag-PEG8-BUF-II-Ag nanobioconjugates, the emulsion without the nanobioconjugate, the commercial product based on fusidic acid (i.e., bacitracin) and bacteria grown in Na$_2$HPO$_4$ buffer. Serial dilutions (1:10) of the bacteria, after the exposure to the treatments, in Na$_2$HPO$_4$ buffer were prepared starting from 100 µL of the supernatant for each treatment. Then, 100 µL of the last dilution were plated in Mueller Hinton agar plates (diameter of 90 mm) and incubated for 18 h at 37°C.
colony forming units (CFU) were counted for each treatment and the bacterial growth percentage was then calculated by taking into account the dilution factor. Bacteria exposed to Na₂HPO₄ buffer was employed as positive control (i.e., bacterial growth of 100%).

**Hemolysis Assay**

Hemocompatibility was assessed by following the ISO 10993-4 standard [59]–[61] for biological evaluation and the ISO 10993-12 [60] standard for sample preparation and reference materials. A sample of 100 µL of each emulsion (O/W emulsions in the presence and absence of the Mag-PEG8-BUF-II-Ag nanobioconjugates and the commercial control containing fusidic acid) was transferred into a 96-well microtiter plate. Then the microplate was centrifuged at 1800 RPM for 5 min to assure a flat surface in each treatment. Each treatment was analyzed in six replicates.

Then, a blood sample was collected from a healthy volunteer in vacutainer blood tubes with EDTA and prepared according to Cuellar et al. [40]. The samples were obtained with the approval of the Ethical Committee at the Universidad de los Andes (minute number 928-2018). Collected blood was centrifuged at 1800 RPM for 5 min. The plasma supernatant was then removed and replaced with 0.9% (w/v) NaCl solution. This process was performed in triplicate and the last supernatant was replaced by PBS (1X) buffer at pH 7.4. A 1:10 dilution of erythrocytes in PBS (1X) was prepared, and 100 µL were pipetted over each treatment. The microplate was then incubated at 37°C for 1h, and 50 µL of the supernatant were transferred to a 96-well microtiter plate and finally read at 450 nm using a microplate reader (Multiskan™ FC Microplate Photometer, Thermo Scientific, FIN). The positive control was an erythrocyte suspension incubated with Triton 100X (100% hemolysis), and the negative control was an erythrocyte suspension incubated with PBS 1X (0% hemolysis).

**Platelet Activation Assay**

The effect of emulsions on platelet activation was evaluated using platelet-rich plasma (PRPs) obtained from blood of a healthy donor, which was previously collected in vacutainer blood tubes supplemented with sodium citrate. PRPs were obtained by centrifugation of the human blood samples at 1000 RPM. Two-fold serially diluted concentrations of the platelets were evaluated starting at 100 % (v/v) down to 0,050 % (v/v) to obtain a calibration curve. Additionally, 100 µL of each treatment (O/W emulsions in the presence and absence of Mag-PEG8-BUF-II nanobioconjugates and the commercial control containing fusidic acid) were placed in a 96-well microtiter plate for exposure of 50 µL of PRP. This was followed by incubation for 30 mins, at 37 °C and 5% CO₂. Then, the platelets supernatant was removed, and the treatments were rinsed with PBS (1X). 100 µL of Triton 100X were added to the treatments and after 15 min of exposure, 50 µL of the supernatant were analyzed via a LDH assay and read at 493 nm in a microplate reader (Multiskan™ FC Microplate Photometer, Thermo Scientific, FI). The platelet activation percentage was obtained by comparing the obtained values for the treatments against the calibration curve.

**Cytotoxicity Assay**

Cytotoxicity assays were performed by the extracts method according to the International Standard ISO 10993-5 [62]. The cell line employed in both assays was HaCaT (ATCC® CRL-2404). An MTT assay was employed to evaluate the cytotoxic effect of the formulated emulsions. 2x10⁴ HaCaT cells were seeded per well in a flat-bottom 96-well microtiter plate and incubated for 24 h at
37°C and 5% CO₂. A 3% (v/v) solution of each treatment was prepared using DMEM culture media without SBF. Each solution was then incubated for 24 h at 37 °C. To obtain the respective extract, each solution was subsequently filtered through cellulose filters (0.22 µm pore size, GE Healthcare, UK). Serial dilutions of the extracts were completed starting at 3% (v/v) down to 0.188% (v/v). The cells were then exposed to 100 µL of each treatment in triplicate and incubated for 24 h under standard conditions. Then, 10 µL of MTT reagent were pipetted to each well and maintained for 2 more hours in the incubator. Finally, the treatments were removed and discarded, and 100 µL of DMSO were added to each well. When all the formed formazan crystals were dissolved in the DMSO, the absorbance was read at 595 nm.

Wound Infection Assay Ex Vivo

Porcine skin was obtained from a local butcher shop and the fatty tissue was carefully removed. Squared samples (1 cm side) of the tissue were cut and a 0.5 cm length superficial wound was made aided by a scalpel. The samples were placed on Petri dishes and washed twice with sterile PBS (1X). Once the wound was made, 2 µL of a 1x10⁸ CFU S. aureus solution was inoculated on the wound along with 2 µL of LB media. The infected skin was incubated for 72 h at 37°C with 10 mL of PBS (1X). The PBS (1X) medium was discarded and replaced with fresh buffer daily. The infected wound was exposed to 50 µL of the treatment (emulsion with 400 µg/mL of the nanobioconjugate and PBS (1X)) dispensed with the aid of an insulin syringe and carefully spread with a spatula. The infection was assessed through, visual checking and confocal microscopy. The effect of the treatments over the infection were monitored through confocal microscopy. For the observation under confocal microscopy the LIVE/DEAD™ BacLight™ Bacterial Viability Kit (L13152) was employed to differentiate live and dead bacterial cells from the porcine tissue.

Results and Discussion

Direct (O/W) emulsions stability

O/W emulsions were prepared and analyzed in the Turbiscan, to determine stability changes. Four O/W emulsions at two dispersed phase concentrations (60% wt. and 70% wt.) and two HLB values (11.79 and 14) were prepared, owing to the high stability reported for concentrated emulsions [49], [50]. The emulsions were monitored daily (Fig. 1A) to identify possible physical instability phenomena such as flocculation or coalescence. After 39 days of analysis, no visible changes were observed in the samples, and the TSI value remained below 4 for the 14 HLB emulsions. On the contrary, the emulsions with a lower HLB value were considered more unstable due to their TSI values above 5. According to Wibowo et al. [48] and Li et al. [63], it is expected that the more concentrated the emulsions, the higher the stability. This has been attributed to a higher presence of small homogeneous drops, which in turn, leads to a more viscous fluid of enhanced stability as reported for colloidal systems containing small amounts of polymeric thickeners. Nonetheless, this was not the case for the emulsion at 70% wt. and HLB 11.79 and, instead, the emulsion at 60% wt. and HLB of 14 exhibited the lower TSI variation. For this reason, it was selected as the most stable formulation for further analyses.

Maintaining constant the dispersed phase concentration and varying the HLB value permitted to distinguish an improvement in the stability behavior of the emulsions. The obtained stability is
explained by the fact that non-ionic surfactants with HLB values above 6 favor the formation of O/W emulsions as a consequence of a larger hydrophilic surface in comparison with that of the hydrophobic tails [64][51]. The hydrophilic heads interact easily with polar substances, which favor water solubilization processes [51]. In this case, Tween 80 (HLB 15) was incorporated at a higher ratio in the surfactant mixture (7:3), thereby increasing the hydrophilic affinity of the emulsion.

After selecting the most appropriate formulation (60% (w/w) and HLB 14), the emulsion was prepared by varying the amount of dispersed nanobioconjugates (400, 330, 200, 100 and 50 µg/mL). The incorporation of the nanobioconjugate was done with a formulation with desirable stability and micro and macromolecular properties, to detect the effect of the nanoparticles when being embedded in the interface of the colloidal system. As observed in Fig. 1B, the emulsion with the lowest concentration of nanobioconjugate display a TSI variation similar to that of the emulsion without the nanobioconjugate, albeit at a TSI value of 1.57 ± 0.06 after day 90. Additionally, the emulsion with the highest amount of nanobioconjugate exhibited a higher increase of the TSI value (6.33 ± 0.15); however, visual inspection revealed no instability phenomena. Despite the measured TSI values, exposure of the emulsions to accelerated stability conditions at 40 °C and 75% humidity for 90 days led to no observable instability phenomena. Moreover, the emulsions in presence of the nanobioconjugate (50 µg/mL) and absence of it exhibited higher stability than that reported for the fusidic acid based product (TSI value of 3 approximately). Additionally, after 90 days of the preparation, all the emulsions showed a pH value close to 7.

Furthermore, this result suggests that the peptide and the nanobioconjugates at 50 µg/mL did not caused significant instability phenomena if compared with the emulsion containing the highest nanobioconjugate concentration. This fact may indicate that, despite remaining in the continuous phase of the O/W emulsions, the nanobioconjugates appear to not interfere with the droplet formation process. This may be explained by the nano size of the nanobioconjugates (aggregates size diameter of 7.84 nm ± 1.61 nm), which is about three orders of magnitude lower than the droplets size (mean hydrodynamic diameter of 1.24 ± 0.001 µm). In addition, low concentrations of the nanobioconjugates impede the coalescence of oil droplets; however, as their concentrations increases, the stability variation also increased up to reach coalescence within a few minutes after the homogenization step (data not shown).

![Fig. 1.](image)
O/W emulsions drop size analysis

Droplets size was measured as the mean hydrodynamic diameter $D_{4,3}$ in a Mastersizer 3000 [65]. Droplets’ size of O/W emulsions prepared at different concentrations and HLB values show a normal distribution (Fig. 2A). Here we obtained a mean hydrodynamic diameter $D_{4,3}$ of 1 µm approximately, which is an appropriate droplet size to avoid precipitation or any other instability phenomena [66]. These results were maintained for all the emulsions monitored even after 95 days.

The favorable results in droplets’ size and their positive impact on the stability of the emulsions may be attributed to the stabilizing role of polymeric matrix formed by the thickener, Carbopol, in the continuous phase, which impedes the mobilization and further aggregation of oil droplets. Carbopol is one of the most known synthetic thickeners and it is frequently used in emulsified systems thanks to the imparted sensorial properties, but also by the provided stability, consistency and droplets homogeneity [67]. Estanqueiro et al. [54] proved that by increasing the amount Carbopol, the droplets’ size decreases, thus, favoring a monodisperse microstructure for the oil droplets.

The emulsions containing different concentrations of the nanobioconjugates exhibited a polydisperse distribution of droplets ranging from 1 µm to 10 µm, while those in their absence showed a unique normal distribution with a particle size of 1.240 ± 0.001 µm (Fig. 2B). This polydispersity may interfere in the stability of the emulsion, as previously discussed for the emulsion with 400 µg/mL of nanobioconjugate, mainly because the greater the oil droplets the greater the attractive forces between the droplets that favor instability phenomena such as coalescence or flocculation. Nonetheless, the particle size of these emulsions (3.950 ± 0.005 µm) remains below that of the commercial treatment (~ 35 µm). This was confirmed by observation under optical microscope (100X) (Fig. S3). Importantly, emulsions with the nanobioconjugate and especially at 400 µg/mL of the nanobioconjugate showed substantial polydispersity.

![Fig. 2. Mean hydrodynamic diameter of O/W emulsions as measured in a Mastersizer 3000 instrument. A) Emulsions at 60% wt., 70% wt., HLB 11.79 and HLB 14. B) Emulsions were prepared with the Mag-PEG8-BUF-II-Ag nanobioconjugate (50 µg/mL and 400 µg/mL) at 60% wt. and HLB 14.](image-url)
Rheological characterization of O/W emulsions

O/W emulsions at both dispersed phase concentrations and HLB values exhibited a pseudoplastic and a shear-thinning behavior (Fig. 3A). By fitting the Power Law to data for each emulsion, showed that the viscosity of the emulsions increased as a function of the dispersed phase concentration and the HLB value. Regarding the oscillatory sweep test (Fig. 3B), the emulsions exhibited the plateau zone where the storage moduli (G’) remained linear for all the emulsions, which indicate the predominance of the elastic response. Furthermore, the G’ value was within the same order of magnitude (~10 Pa), although, the higher the concentration and the HLB value, the higher the storage moduli (Fig. 3A). On the contrary, the loss moduli (G’’) showed a non-linear behavior (Table 2), with variations of about an order of magnitude between samples. These results indicate that the emulsion will be easily spread over the affected area, and it will be retained in the tissue thanks to it recovers its viscosity after being subjected to shear stress (thixotropic character).

Table 2 Rheological parameters of O/W emulsions at two different concentrations of dispersed phase and two different values of HLB. The values of m and n were obtained from the power law, R is the curves fit to the power law, while G’ and G’’ correspond to the mean values of each modulus in the curve.

<table>
<thead>
<tr>
<th>Emulsion</th>
<th>R</th>
<th>m</th>
<th>n</th>
<th>G’</th>
<th>G’’</th>
</tr>
</thead>
<tbody>
<tr>
<td>60% wt., HLB 11.79</td>
<td>0.957 ± 0.217</td>
<td>131.45 ± 35.07</td>
<td>0.206 ± 0.036</td>
<td>344.23 ± 7.194</td>
<td>16.825 ± 3.881</td>
</tr>
<tr>
<td>60% wt., HLB 14</td>
<td>0.953 ± 0.186</td>
<td>182.49 ± 31.47</td>
<td>0.164 ± 0.014</td>
<td>573.26 ± 17.82</td>
<td>23.372 ± 6.057</td>
</tr>
<tr>
<td>70% wt., HLB 11.79</td>
<td>0.881 ± 0.383</td>
<td>213.37 ± 10.06</td>
<td>0.135 ± 0.003</td>
<td>602.37 ± 11.40</td>
<td>25.058 ± 15.73</td>
</tr>
<tr>
<td>70% wt., HLB 14</td>
<td>0.872 ± 0.698</td>
<td>252.76 ± 35.95</td>
<td>0.120 ± 0.012</td>
<td>782.31 ± 14.22</td>
<td>28.22 ± 15.82</td>
</tr>
</tbody>
</table>

Fig. 3. Rheological analyses of O/W emulsions at 20°C. The emulsions were prepared with the Mag-PEG8-BUF-II nanobioconjugate at 60% wt, 70% wt, HLB 11.79 and HLB 14. A) Stress τ vs. shear-rate and viscosity η vs. shear-rate (hollow points) at 0.1 - 200 s⁻¹. B) Frequency sweep analyses of O/W emulsions performed at 20°C, 1% strain and 0.01 – 1 Hz (hollow points correspond to G’’).

Considering that the most stable emulsion was the formulation of 60 % wt. and HLB 14, two different concentrations of nanobioconjugate were tested to determine whether their presence might alter the viscosity and the rheological behavior of the emulsions. Fig. 4A shows that even though there exists a visible decrease in the viscosity and shear-stress of the emulsion containing 400 µg/mL,
the pseudoplastic and shear-thinning behavior remain largely unaltered. This agrees well with previous reports by Sharkawy et al. [68] and Bhagavathi et al. [69] who demonstrated that emulsions containing nano-systems, such as chitosan nanoparticles and multiwalled carbon nanotubes, preserve their pseudoplastic behavior. This characteristic is desirable to facilitate the application of the treatment over the affected skin. This dependence is also related to the presence and the type of the thickening agent. Moreover, the distinct elastic and viscous properties exhibited by the emulsions are influenced by this factor [70], [54] and by the high concentration of the oil [50], [63].

Contrary to the control with fusidic acid, the formulated emulsion in the presence and absence of nanobioconjugates are thixotropic, which means that they recover their viscosity after being exposed to high shear rates. Regarding the oscillatory sweep, it was observed that both $G'$ and $G''$ moduli decrease when increasing the amount of nanobioconjugates in the continuous phase; however, the elastic modulus is the predominant one (Fig. 4B, Table 3), which indicates that the product will not flow rapidly unless an external force is applied. These results agree well with those obtained by Gilbert et al. for synthetic thickeners where they demonstrated that the viscoelastic behavior is attributed to the ability of synthetic textural agents to form gel structures, which permits the emulsions to be more resistant to structural breakdown [67]. Here, this was the case Carbopol but not for xanthan gum where more fluid emulsions were obtained with a higher sensitivity to high shear-rates and viscosity and moduli values an order of magnitude lower than the exhibited by the emulsions prepared with Carbopol (data not shown). Once more, our results seem consistent with those previously reported by different authors [54], [57], [67].

**Table 3** Rheological parameters of O/W emulsions (60% wt. and HLB 14) at two different concentrations of the Mag-PEG8-BUF-II-Ag nanobioconjugate. The values of $m$ and $n$ were obtained from the power law, while $G'$ and $G''$ correspond to the mean values of each module in the curve.

<table>
<thead>
<tr>
<th>Emulsion</th>
<th>$R$</th>
<th>$m$</th>
<th>$n$</th>
<th>$G'$</th>
<th>$G''$</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 µg/mL</td>
<td>0.942 ± 0.007</td>
<td>161.21 ± 4.96</td>
<td>0.1667 ± 0.003</td>
<td>445.35 ± 2.222</td>
<td>16.863 ± 0.754</td>
</tr>
<tr>
<td>400 µg/mL</td>
<td>0.95 ± 0.000</td>
<td>115.48 ± 0.12</td>
<td>0.1889 ± 0.003</td>
<td>314.19 ± 2.562</td>
<td>14.922 ± 0.204</td>
</tr>
<tr>
<td>Control</td>
<td>0.933 ± 0.009</td>
<td>235.98 ± 5.58</td>
<td>0.1480 ± 0.001</td>
<td>663.76 ± 13.43</td>
<td>34.073 ± 2.888</td>
</tr>
<tr>
<td>Fusidic Acid</td>
<td>0.4724</td>
<td>7.7221</td>
<td>0.4094</td>
<td>446.67</td>
<td>455.89</td>
</tr>
</tbody>
</table>
Fig. 4. Rheological analysis of O/W emulsions performed at 20°C. The emulsions were prepared with the Mag-PEG8-BUF-II-Ag nanobioconjugate (50 µg/mL and 400 µg/mL) at 60% wt and HLB 14. The emulsion without the nanobioconjugates and a commercial control based on fusidic acid were employed as controls. A) Stress $\tau$ vs. shear-rate and viscosity $\eta$ vs. shear-rate (hollow points) at 0.1 - 200 s$^{-1}$. B) Frequency Sweep analysis of O/W emulsions performed at 20°C, 1% strain and 0.01 - 1Hz (hollow points correspond to G'').

Scaling-up Process of the emulsion at bench scale

Considering the results previously presented, the direct emulsion 60% wt. and HLB 14 was selected for scaling-up from laboratory to bench scale. For this purpose, we explored the preparation of three different amounts of emulsions (100 g, 1000 g and 3000 g) (Fig. 5). This was accomplished by taking into account several parameters such as the impellers diameters relation [71], the final height of the emulsion contained in the mixing container, and the impeller clearance [49], [72]. As part of the scaling-up process, it was necessary to design the impeller geometry according to the appropriate specifications. In this procedure, the tip velocity, which refers to the velocity that the particles experienced in the tip of the impeller [49], [73], was kept constant to ensure that the oil droplets were incorporated under the same conditions.

The scaling-up of processes involving Newtonian fluids rely on keeping constant non-dimensional numbers, such as Reynols (Re) and Froude (Fr) to obtain the new mixing equipment dimensions and conditions [71]. However, for non-Newtonian fluids, such as the topical emulsions developed here, the viscosity changes with the variation of the shear rate, which makes the estimation non-dimensional numbers impractical [71], [74]. In this regard, calculating the non-dimensional numbers for non-Newtonian fluids, involves using modified equations where rheological indexes are incorporated [74], [75]. Alternatively, a more accurate scaling-up parameter is the tip-velocity due to its independency with respect to the rheology of the fluid [49], [76]. Regarding the impeller selection, it is necessary to consider its pumping capacity (widely reported for different geometries) [74], [76], [77], and the type of flow produced (i.e., axial or radial) because these parameters largely define the vortex formation and the obtained flow regime [72], [74].
Fig. 5. Pictures of the 70%wt. HLB 14 O/W emulsions prepared at different scales. From left to right: 100 g, 1000 g and 3000 g.

The flow sweep comparing the three preparations showed that all the emulsions exhibited pseudoplastic and shear-thinning behavior; however, the consistency and flow indexes varied from one emulsion to the other, that is to say that the greater the quantity prepared, the smaller is the consistency index (Fig. S4A). This phenomenon was also observed in the oscillatory tests where, despite the linearity of G' for the three preparations, the value of the modulus decreased with the amount of emulsion prepared. The behavior of the G” modulus show no clear trend for the different samples (Fig. S4B). The significant differences observed for the 3000 g sample can be attributed to changes in the vessel geometry and dimensions, and particularly the height/diameter (T/H) ratio for the tank that approached ~ 0.65; however, it has been recommended a 1 ratio to avoid dead zones.

Regarding to the droplet size, the distribution of the 1000 g and 3000 g preparations is similar and the D[4,3] is 1.25 µm and 1.21 µm, respectively (Fig. S5A). Despite the differences in the volume density percentage with respect to the 100 g preparation, the order of magnitude of the obtained sizes is about the same in all the samples. This confirms that the performance of the scaled-up systems is comparable to that at the lab scale [65]. Regarding to the final product properties, the emulsion prepared at the bench scale will exhibit a lower viscosity leading to sensorial differences when comparing with the emulsion prepared at the laboratory scale. In addition, stability in time may vary from one scale to the other, owing to the pumping performance of the assembly. For this reason, it is important to maintain the scaling-up parameters and dimensions, and consider the implementation of accessories like baffles, between the scales in order to avoid differences or variation of properties like spreadability, retention time and stability.

Antibacterial assay

Previous studies developed by Perez J. et al. [24], determined that the Mag-PEA-BUF-II nanobioconjugate exhibited a great capability of escaping endocytic internalization pathways, however, the bacterial growth was ~50% of that of the free peptide at a nanobioconjugates’ concentration of 1 mg/mL. Furthermore, it was evidenced that the cytotoxic effect on human cells increased when increasing the concentration of the nanobioconjugates. In order to boost the antimicrobial effect of the designed treatment, Mag-PEA-BUF-II nanobioconjugates were enhanced by replacing the PEA by a heterobifunctional PEG (PEG8) and a silver shell on the surface of the nanobioconjugates to obtain the Mag-PEG8-BUF-II-Ag nanobioconjugates. Additionally, in this
assay the treatment employed was the undiluted emulsion containing 400 µg/mL of the Mag-PEG8-BUF-II-Ag. The antibacterial assay was performed with a Gram-positive strain, *Staphylococcus aureus*, and a Gram-negative strain, *Escherichia coli*.

7.1x10⁶ ± 3.8x10⁶ CFU were obtained from *S. aureus* suspended in the Na₂HPO₄ buffer, this value was assumed to correspond to a bacterial growth of 100%. Fig. 6 shows that no bacterial growth was detected in the presence of the topical treatment containing 400 µg/mL of the nanobioconjugate Mag-PEG8-BUF-II-Ag. This result can be visualized in Fig. 7. Additionally, a bacterial growth of 78.4% was obtained with the topical treatment in the absence of nanobioconjugates and of 140% in the presence of commercial control made of fusidic acid. The CFUs were 5.6x10⁶ ± 1.7x10⁶ and 1x10⁷ ± 2.8x10⁶, respectively. These results are promising as they demonstrated that our treatment is superior to one of the most popular commercial treatments. However, the obtained result might be related to the slow diffusion rate of the commercial treatment, which might take up to 2 h for a complete inhibition of *S. aureus* [10], [44]. Considering that our topical treatment inhibits bacterial growth in a short period of time (2 h), the product might have a potential for the clinical treatment of methicillin resistant strains of *S. aureus* (MRSA).

The positive control of *E. coli* was obtained at a 1.15x10⁷ ± 3.4x10⁶ CFU, which was assumed to correspond to a bacterial growth of 100%. As observed for *S. aureus*, the emulsion containing the nanobioconjugates completely inhibited the bacterial growth of *E. coli*; nonetheless, in the treatments exposed to the emulsion without the nanobioconjugates and the commercial control, the bacterial growth was higher than for the positive control. This is most likely due to the presence of mineral oil, which has been reported to preserve different bacterial strains for long periods, including *E. coli* [78]. Taken together, the antibacterial activity results of the topical treatment containing the Mag-PEG8-BUF-II-Ag nanobioconjugates hold much promise as a novel alternative to mupirocin- and fusidic acid-based topicals to combat nosocomial and community acquired infections [10], [13], [44].

![Fig. 6](image.png)

Fig. 6. Antibacterial activity assay performed with *S. aureus* and *E. coli*. The emulsion tested was prepared with the Mag-PEG8-BUF-II-Ag nanobioconjugate (400 µg/mL) at 60% wt. and HLB 14. The emulsion without the nanobioconjugates and a commercial control based on fusidic acid were employed as controls. The positive control consisted of bacteria grown in Na₂HPO₄ buffer.
Fig. 7. Images of the antibacterial activity assay performed with A. *S. aureus* and B. *E. coli*. The emulsion tested was prepared with the Mag-PEG8-BUF-II-Ag nanobioconjugate (400 µg/mL) at 60% wt and HLB 14; no CFU were identified for this treatment. The emulsion without the nanobioconjugates (Control) and a commercial control based on fusidic acid were tested for comparative purposes.

**Hemolysis assay and platelet activation assay**

Considering that the formulated topical treatment based on Mag-PEG8-BUF-II-Ag nanobioconjugates is intended to be applied superficially on skin infections, the treatment was analyzed undiluted. Previous results of diluted topicals from 50 % (w/v) down to 1.65 % (w/v) indicated hemolysis percentages below 5 % (data not shown). However, platelet aggregation varied from 50 % to 80 % at some concentrations, and consequently, platelet activation was tested instead.

**Fig. 8A** shows that the O/W emulsion, with different concentrations of the nanobioconjugate, exhibited a hemolysis percentage ranging between 15 % and 20 %. Similar results were obtained for the emulsions without nanobioconjugate and for the commercial treatment based on fusidic acid. Despite obtaining a hemolysis percentage above 5 % (which is the limit indicated in the ISO 10993-4 standard to consider that a medical device is not hemolytic [61]), the hemolysis percentage obtained is acceptable considering that the products are not intended for open wounds [79], [80].

The platelet activation assay allowed evaluating if the platelets activated or adhered after coming in contact with the topical. **Fig. 8B** showed that the platelet activation ranged between 10 % and 20 % for all the treatments tested. As mentioned before, the emulsions will not be in contact with open wounds, therefore the chances of thrombus formation are highly limited.
Fig. 8. Biocompatibility assays. The tested topicals were prepared with the Mag-PEG8-BUF-II-Ag nanobioconjugate (400, 330, 200 and 100 µg/mL) at 60% wt and HLB 14. The emulsion without the nanobioconjugates and a commercial control based on fusidic acid were employed as controls. In the hemolysis assay Triton 100X was used as the positive control while PBS 1X was the negative one. In the platelet activation assay poor platelets plasma (PPP) was employed as the negative control and Thrombin as the positive control.

Cytotoxicity assay

The cytotoxic effect of the emulsions was assessed through an indirect assay based on extracts. Fig. 9 indicates that at concentrations greater than 1.5 % (v/v) both emulsions are highly cytotoxic. This result may be associated to the fact that the emulsions contain a high contents of oil phase, thus, when diluted in the buffers such components tend to accumulate at the surface of the solution. As a result, when cells are exposed to the treatments, the formed oil layer impedes the appropriate air exchange, which is detrimental to cells survival (Fig. S6). Our results agree with those of Okur et al. [81], Coelho De Bari et al [82], and Placzek et al. [83] who developed therapeutic emulsions for topical, oral or parenteral administration. The authors reported cell viability of murine or human cell lines above 70% when exposed to emulsions at concentrations below 4% (v/v).

Emulsions are widely used in the pharmaceutical field, however highly viscous emulsions, like creams, have found application in skin cancer treatment due to their versatility in the incorporation of different active agents and materials [84]. Also, when essential oils and surfactants (such as polysorbate 80) are used to prepare them, they exhibit significant cytotoxicity towards the cancer cells and tumors [85][82]. Despite reducing cell viability of 3T3 cultures, polysorbate 80 has been incorporated into several skin care commercial products, body lotions and shampoos. Moreover, some antibacterial topical treatments contain low concentrations of this component combined with other molecules to improve their colloidal stability [86].
A first approach to test the topical treatment in an infected wound was approached *ex vivo* in porcine skin. Two different times of infection were tested to determine the most accurate time of incubation to mimic an *in vivo* infected tissue. **Fig. 10** shows color changes around the area exposed to $1 \times 10^7$ CFU at 24 h and 48 h, which may indicate the bacterial growth upon inoculation. Nonetheless, the infection in the sample treated on day 0 was more noticeable and seemed to be more homogeneous than the sample infected 24 h after incubation. In this experiment it is important to consider that the tissue exhibited an intrinsic change in color and appearance due to the temperature conditions of (37°C), and the lack of cell culture media for the maintenance of keratinocytes and fibroblasts present in the skin tissue. Additionally, 24 h after the application of the topical treatment, the emulsion diffused along the infected tissue, which indicates that the nanobioconjugates entrapped in the emulsion will most likely reach the proliferated bacteria cells and, therefore, reduce or completely inhibit their viability. From this assay, we concluded that further studies should be conducted in an infected wound without previous incubation of the tissue and to apply the treatment 72 h after the infection.

In our study, the use of porcine skin samples is a first step towards the study of the bactericidal feature of the formulated topical treatment in a more complex system, as well as possible secondary effects of the tissue when exposed to the emulsion. It has been reported by different authors like Rosselle et al. [87] and Raveendran et al. [88], among others [89]–[91], wound infected assays performed in pigskin as preliminary experiments to determine the antibacterial effect or the antibacterial response of novel treatments and medical techniques. These experiments allowed the authors to determine patterns, through visual analysis or more sophisticated techniques like microscopy, about the effect of their treatments before proceeding with *in vivo* experiments. Despite the tissue is not treated as an explant, it is a valuable tool to recreate infected wounds to further study antibacterial properties of a material. Nonetheless, further analysis will consider the use of a 3D human skin model that offers a more reliable platform for the study of the interaction of the bacteria and the emulsion with a complex system made of skin human cells.
Fig. 10. Wound infection performed with *S. aureus* (1x10^7 CFU) on pigskin. Blue circles indicate the infected area 48 h after being inoculated; the upper image correspond to the sample infected at day cero and lower image correspond to the sample incubated 24 h before inoculation.

Conclusion

Finally, through the integrated process design, that includes the macroscopic and microscopic properties of a product, it has been possible to develop an emulsion that, combined with the antimicrobial vehicle Mag-PEG8-BUF-II-Ag nanobioconjugates, may be potentially used as topical treatment to treat and recover the skin tissue affected by the infections caused by *S. aureus*. Rheology, particle size and stability, had been of great importance in the selection of the most appropriate formulation. In agreement with this, the direct emulsions were a stable colloidal system, and its reproducibility has been demonstrated at different scales. The formulated emulsion containing the novel nanobioconjugates Mag-BUF-II-Ag demonstrated to be highly effective in avoiding the bacterial growth of *S. aureus* and *E. coli* wild type strains. Furthermore, despite hemolysis and platelet aggregation were upper 5%, the results of the designed product were like those exhibited by the commercial control. In addition, the emulsions do not represent a risk for the user, due to their use is superficial owing that they are intended to treat patients in the initial stage of the infection. Finally, it was identified a possible route to evaluate the bactericidal effect of the topical treatment over an infected wound by using porcine skin instead of *in vivo* tests.

The future work of this project includes a deeper study and analysis of the cytotoxic effect of the emulsions tested through *in vitro* assays and in a 3D human skin model where the co-infection
will be recreated. This experiments will be conducted under the supervision and guidance of “Grupo de Investigación en Nanomateriales, Ingeniería de tejidos y Bioimpresion” (GINIB) of the Biomedical Engineering Department at Universidad de los Andes.

Acknowledgments

The authors would like to thank to the Biomedical Engineering and the Chemical Engineering and Food Departments of Universidad de los Andes for their support and for providing access to their laboratories. Special gratefulness to the member of Grupo de Investigación en Nanobiomateriales, Ingeniería Celular y Bioimpresión (GINIB) and Grupo de Diseño de Productos y Procesos (GDPP) for their continued support throughout the development of this project. Finally, the financial support by MINCIENCIAS grant 689-2018 is gratefully acknowledged.

Bibliography


## Supplementary Material

### Tables

**Table S1.** Parameters and dimensions used in the scale-up process at three different scales (100 g, 1000 g and 3000 g).

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Parameter</th>
<th>Scale-up Process Dimensions [cm]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Recipient</strong></td>
<td></td>
<td><strong>100g</strong></td>
</tr>
<tr>
<td>T</td>
<td>Inner Diameter</td>
<td>6,62</td>
</tr>
<tr>
<td>H</td>
<td>Height</td>
<td>9,98</td>
</tr>
<tr>
<td><strong>Impeller</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>Total Diameter</td>
<td>5,30</td>
</tr>
<tr>
<td>L</td>
<td>Blade Length</td>
<td>1,77</td>
</tr>
<tr>
<td>W</td>
<td>Blade Height</td>
<td>2,02</td>
</tr>
<tr>
<td>B</td>
<td>Width</td>
<td>0,30</td>
</tr>
<tr>
<td>A</td>
<td>Axis Width</td>
<td>3,64</td>
</tr>
<tr>
<td>S</td>
<td>Shaft Diameter</td>
<td>7,74</td>
</tr>
<tr>
<td>°</td>
<td>Angle of Declination</td>
<td>60</td>
</tr>
<tr>
<td><strong>Assembly</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Clearance</td>
<td>2,65</td>
</tr>
<tr>
<td>Z</td>
<td>Liquid Height</td>
<td>6,62</td>
</tr>
</tbody>
</table>

*The inner diameter and the height of the recipient were measured in the laboratory.*

*The impeller diameter (D), the blade height (W), the liquid height (Z) and the clearance (C) were calculated with the following relations:

\[
\frac{D}{T} = 0.8, \quad \frac{W}{L} = 0.5, \quad \frac{Z}{T} = 1, \quad \frac{C}{T} = 0.4, \quad \text{Equation S1}
\]
Fig. S1. Procedure and reactions involved in the synthesis of the MNP-BUF-II-Ag nanobioconjugates.
**Fig. S2.** Scaling-up assembly. **A**) $60^\circ$ pitch-blade impeller dimensions employed in the scaling-up process (3000 g). **B**) Impeller location in the recipient.

**Fig. S3.** Optical microscope observation, 100X (scale bar 5µm), of the O/W emulsions at 60% wt. and HLB 14 prepared with the nanobioconjugate Mag-PEG8-BUF-II-Ag (50 µg/mL and 400 µg/mL) 90 days after their preparation. Orange arrows indicate larger droplets.
Fig. S4. Rheology of O/W emulsions prepared at three different scales at 60% wt and HLB 14. A) Shear stress \( \sigma \) (solid symbols) and viscosity \( \eta \) (hollow symbols) performed at 20°C, 0.1 - 200 s\(^{-1}\) and 200 – 0.1 s\(^{-1}\). B) Frequency Sweep analysis at 20°C, 1% strain and 0.01 - 1Hz.

Fig. S5. A) Mean hydrodynamic diameter and B) stability index through time of O/W emulsions prepared at different scales (100 g, 1000 g, 3000 g).
Fig. S6. Image of a 24 well-plate after the exposition of HaCaT cells to a 50% v/v solution of the emulsion containing the nanobioconjugate Mag-BUF-II-Ag (400 µg/mL). The orange arrows indicate the oil phase after removing the DMEM media (upper) and the oil phase in the surface of the DMEM media (down).