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Vancomycin-loaded nanobubbles: A new platform for controlled antibiotic delivery against methicillin-resistant Staphylococcus aureus infections

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(Article begins on next page)

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Abstract: Vancomycin (Vm) currently represents the gold standard against methicillin-resistant Staphylococcus aureus (MRSA) infections. However, it is associated with low oral bioavailability, formulation stability issues, and severe side effects upon systemic administration. These drawbacks could be overcome by Vm topical administration if properly encapsulated in a nanocarrier. Intriguingly, nanobubbles (NBs) are responsive to physical external stimuli such as ultrasound (US), promoting drug delivery. In this work, perfluoropentane (PFP)-cored NBs were loaded with Vm by coupling to the outer dextran sulfate shell. Vmloaded NBs (VmLNBs) displayed ~300 nm sizes, anionic surfaces and good drug encapsulation efficiency. In vitro, VmLNBs showed prolonged drug release kinetics, not accompanied by cytotoxicity on human keratinocytes. Interestingly, VmLNBs were generally more effective than Vm alone in MRSA killing, with VmLNB antibacterial activity being more sustained over time as a result of prolonged drug release profile. Besides, VmLNBs were not internalized by staphylococci, opposite to ${\tt Vm}$ solution. Further US association promoted drug delivery from VmLNBs through an in vitro model of porcine skin. Taken together, these results support the hypothesis that proper Vm encapsulation in US-responsive NBs might be a promising strategy for the topical treatment of MRSA wound infections.

Torino, Italy: 14th March 2017

To the Editor

of the International Journal of Pharmaceutics

Dear Editor,

please find attached here the revised version (both marked and clean copies) of our research article titled "Vancomycin-loaded nanobubbles: a new platform for controlled antibiotic delivery against methicillin-resistant *Staphylococcus aureus* infections".

As requested, the manuscript was implemented according to the reviewer's suggestions and all the references were modified according to the journal's author guidelines. Following the reviewer's comments, the image quality was improved for all figures. A rebuttal letter containing our replies to the author(s)'s comments is also attached.

We sincerely hope that you will find the revised version of the manuscript acceptable for publication by the International Journal of Pharmaceutics.

We are looking forward to receiving your feedback.

Kind regards

Prof. Roberta Cavalli

IJP AUTHOR CHECKLIST

Dear Author,

It frequently happens that on receipt of an article for publication, we find that certain elements of the manuscript, or related information, is missing. This is regrettable of course since it means there will be a delay in processing the article while we obtain the missing details.

In order to avoid such delays in the publication of your article, if accepted, could you please run through the list of items below and make sure you have completed the items.

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Reviewers' comments:

Reviewer #1: Comments:

1. The quality of figures should be greatly improved, especially the graphical abstract, Figure 3 and 6.

We apologize for such an issue. According to the reviewer's suggestion, the image quality was improved for all figures.

2. In line 159, the section 2.2.1., How many ml of dextran sulfate aqueous solutions were added into the Vm solution? What is the solvent of Vm solution? Water or other organic solvents? When centrifuge the unbound Vm, how much is the centrifugal speed? And the centrifugal time also should be provided.

One ml of dextran sulfate aqueous solution at increasing concentrations was added into 1 ml of vancomycin aqueous solution. The centrifugal speed used was 20000 rpm for 15 minutes. The manuscript was implemented with such information (Materials&Methods section, par. 2.2.1).

3. For preparing pre-emulsion containing Epikuron[®] 200, palmitic acid and PFP, how much g/mg of PFP was used? To prepare polymeric NBs, How many ml of dextran sulfate aqueous solution was added into the PFP emulsion?

The amount of PFP and dextran sulfate used for each nanobubble formulation were 500 μ L and 350 μ L, respectively. Such information was added in the text (Materials&Methods section, par. 2.2.2).

4. In line 199, for the TEM observation, the type and brand for the TEM equipment should be provided in the text.

The type and brand of the instrument used for TEM analyses (Philips CM10 (Eindhoven, NL)) were added in the text (Materials&Methods section, par. 2.3.1).

5. To measure the loading capacity, the VmLNBs solution was sonicated and centrifuged, and then the supernatant was analyzed. How to validate the VmLNBs were completely broken? Why not use the organic solvent to destroy the structure of the VmLNBs?

The parameters of the used freeze-drying process are severe to maintain the integrity of the nanostructure in the absence of any cryoprotectors. Preliminary experiments were carried out to evaluate by optical microscopy the nanobubble structure and to set a protocol suitable for determining the loading capacity. The manuscript was modified accordingly (Materials&Methods section, par. 2.3.5).

6. In line 285, the unit of centrifugal speed was g, in line 230, the unit is rpm. The author should check them. Some similar expressions also should be uniformed, such as mL and microL.

All units of centrifugal speed as well as those indicating microliters were uniformed throughout the full text.

7. The viscosity of VmLNBs was higher than that of NB. The reason should be explained in the section of "Discussion". Does the change of viscosity affect loading capacity, encapsulation efficiency, physical stability, Vm release, and permeation efficiency?

We apologize for the typing mistake concerning the viscosity value of blank NB formulations. We determined again the viscosity using the Ubbelhode capillary viscosimeter to confirm the data. The viscosity of all NB formulations (i.e. blank NBs, VmLNBs, fluorescent NBs, and fluorescent VmLNBs) did not show any significant changes. A specific sentence was added in the text (Results section par. 3.1).

8. Table 2 can be incorporated in Table 1.

According to the reviewer's suggestion, Table 2 was incorporated in Table1.

Reviewer #2: The development of novel systems for antibiotics is in its infancy as compared to other disease conditions and is receiving increasing interest in the literature. Whilst several nanosystems are being reported for vancomycin, few, if any have been with nanobubbles. Further, transdermal delivery of nanoencapsulated antibiotics is an emerging research area. This paper describes the encapsulation of vancomycin into nanobubbles for ultrasound mediated drug release and also to bypass the stratum corneum to optimize the treatment of wound infections. This proof of concept study is well designed and the potential of this delivery system is demonstrated. The paper is well written with some minor recommendations:

1. Images of the nanobubbles show one with a single nanobubble and another with 2. Ideally an image showing a population representation should be considered.

A TEM image showing a population representation of VmLNBs was added in the Supplementary Information.

2. There are several inconsistencies in the referencing style which need to be corrected.

All the references were modified according to the journal's author guidelines.

3. Pg 18, Line 368. The last sentence is incorrect and needs to be rewritten.

According to the reviewer's suggestion, we changed the sentence as follows: "As shown in Figure 3, the drug resulted much more stable from a chemical point of view when properly incorporated in the nanocarrier (VmLNBs) than as such in solution." (Results section, par. 3.2)

Reviewer #3: Manuscript IJP-D-16-02774 "Vancomycin - loaded nanobubbles: a new platform for controlled...." by Argenziano et al. describes the fabrication, characterization and release capability of polymer shelled droplets loaded with vancomycin.

The manuscript should be implemented according to the following comments:

1) It should be specified whether PFP is liquid.

Perfluoropentane is a perfluorocarbon with a boiling point of 29°C, hence liquid at room temperature. The use of PFP allows liquid droplet generation at room temperature. Then, PFP in nanodroplets can be activated by an external stimulus, like ultrasound, by means of a mechanism called acoustic droplet vaporization, causing the droplet to become a bubble. The sentence was added in the manuscript (Introduction section, lines 117-120).

2) Term "nanobubbles" can be misleading. At room temperature the core of the particles is liquid PFP. Therefore nanobubbles does not describes correctly such particles. It would be more proper the term "nanodroplets" or "nanovescicles"

The formulation is referred to as "nanobubbles" for sake of simplicity (to distinguish them from so-called decafluoropentane-containing nanodroplets, already patented by our group; see Introduction section for more details about those nanodroplets) but we acknowledge that, prior to the application of ultrasound, it would be more precise to use the term "nanodroplets" when the core is constituted of perfluoropentane. This clarification was included in the text (Introduction section, lines 129-132).

3) Figures are not numbered and are very low in resolution (including the graphical abstract). Sometimes they are not readable.

We apologize for the low quality of figures. According to the reviewer's suggestion, the resolution of all figures was improved. Also, Figures were numbered in accordance to legend numbers.

4) Scheme of the particle differs from the particle description of the graphical abstract in the position of vancomycin, tethered to the external surface of the particle and in the particle shell, respectively.

Vancomycin is included in the polysaccharide shell. For clarity, we modified Figure 1.

5) Viscosity measurements obtained by capillary viscosimetry should be defined. With an Ubbelhode capillary viscometer a relative viscosity, a specific viscosity, an intrinsic viscosity can be obtained. Which one is reported ? All of them have different dimensions from the reported one, i.e. cP. Relative (to solvent) and specific viscosities are dimensionless, intrinsic viscosity has dimension of an inverse of concentration.

With the Ubbelhode capillary viscometer, the time required for the nanosuspension to flow through a capillary of a known diameter of a certain factor (K) between two marked points was measured. By multiplying the time taken, by the factor of the viscometer (0.105), the kinematic viscosity was obtained. The dynamic viscosity was obtained by multiplying kinematic viscosity by density. The cP is the unit of dynamic viscosity in the metric CGS (centimeter-gram-second) system.

6) Vancomycin permeation study puzzled me a lot. The experiment should be conducted at osmotic conditions. To avoid Donnan effects with a charged not diffusible solute, i.e. nanobubbles, a suitable diffusible ionic strength should be used on both the compartments separated by the membrane. According to the given description NaCl 0.9 % w/w has been added only on one compartment. In this conditions other, not controlled contributions affects the diffusion process of vancomycin. In the description of the set up, the concentration of nanobubbles is not reported.

We apologize for the inaccurate description of the experimental setup. For *in vitro* permeation studies, NB samples were prepared in saline solution (NaCl 0.9% w/v). The concentration of NBs in the donor phase was 1×10^{12} NBs/ml. All information was added to the text (Materials&Methods section, par. 2.2.2 and 2.5).

7) When ultrasound are applied, it is important to check the behaviour of the "nanobubbles" (nanodroplets) in order to frame the enhanced release. Do "nanobubbles" (naonodroplets) undergo acoustic droplet vaporization? This effect is known to transform droplets into bubbles, thus changing the release of the payload.

Nanobubbles were observed by US standard imaging (MyLab ESAOTE instrument) and they showed a good scattering response, either in the absence or presence of vancomycin. Further investigations are needed to check whether actual vaporization occurred. With regard to the drug release, preliminary experiments showed an enhanced release kinetics after US application.

8) Pg 6 line 115: PFP is liquid or gas?

Perfluoropentane is liquid at room temperature, having a boiling point of about 29 °C. Therefore, it is gaseous at body temperature (37°C) as such.

9) Pg 15 line 332: why in confocal microscopy imaging, bacteria are dried ?

The drying of bacteria is a step necessary for their staining. After smearing of bacteria on the glass-slide, every staining procedure considers that bacteria have to be air-dried to fix them on the slide and to avoid the subsequent rinsing of the smear during staining procedure, as well as to allow the sample to more readily take up stain(s).

10) Vancomycin hydrochloride is not mentioned in the Material section.

Vancomycin hydrochloride was from Sigma-Aldrich (St Louis, MO). Therefore, it falls into the general sentence "All materials were from Sigma-Aldrich, St Louis, MO, unless those indicated as follows" (first sentence of par. 2.1 in Materials&Methods section).



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2	methicillin-resistant Staphylococcus aureus infections.
3	
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5	Rossana Gulino ⁴ , Amina Khadjavi ^{4,5} , Rita Spagnolo ¹ , Vivian Tullio ² , Giuliana Giribaldi ⁴ , Caterina
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Vancomycin-loaded nanobubbles: a new platform for controlled antibiotic delivery against

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- 23 Abstract
- 24

Vancomycin (Vm) currently represents the gold standard against methicillin-resistant Staphylococcus 25 *aureus* (MRSA) infections. However, it is associated with low oral bioavailability, formulation stability 26 issues, and severe side effects upon systemic administration. These drawbacks could be overcome by 27 Vm topical administration if properly encapsulated in a nanocarrier. Intriguingly, nanobubbles (NBs) 28 are responsive to physical external stimuli such as ultrasound (US), promoting drug delivery. In this 29 work, perfluoropentane (PFP)-cored NBs were loaded with Vm by coupling to the outer dextran sulfate 30 shell. Vm-loaded NBs (VmLNBs) displayed ~300 nm sizes, anionic surfaces and good drug 31 encapsulation efficiency. In vitro, VmLNBs showed prolonged drug release kinetics, not accompanied 32 by cytotoxicity on human keratinocytes. Interestingly, VmLNBs were generally more effective than 33 Vm alone in MRSA killing, with VmLNB antibacterial activity being more sustained over time as a 34 result of prolonged drug release profile. Besides, VmLNBs were not internalized by staphylococci, 35 opposite to Vm solution. Further US association promoted drug delivery from VmLNBs through an in 36 *vitro* model of porcine skin. Taken together, these results support the hypothesis that proper Vm 37 encapsulation in US-responsive NBs might be a promising strategy for the topical treatment of MRSA 38 wound infections. 39

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41 Key words

- 42 Nanobubbles; vancomycin; methicillin-resistant *Staphylococcus aureus*; ultrasound; prolonged release.
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47 **1. Introduction**

48

Chronic wounds fail to proceed through timely regulated and interrelated processes to restore anatomical and functional integrity of the injured tissues (Lazarus et al., 1994) such as diabetic feet, bedsores, and venous ulcers (Markova et al., 2012). To date, these types of wounds are considered like a silent epidemic, affecting a large fraction of the world population and posing a major gathering threat to the public health and economy of all developed countries (Daeschlein, 2013). Hospitalized patients are at particular risk, especially those suffering from diabetes, human immunodeficiency virus or other immune disorders, as well as those undergoing chemotherapy (Payne et al., 2008).

Beyond delayed healing processes due to different factors (hypoxia, persistent inflammation, and 56 altered balances between tissue remodelling proteinases and their inhibitors), chronic wounds are often 57 worsened by microbial infections (Gurusamy et al., 2013). Among the bacteria responsible for skin 58 infection, *Staphylococcus aureus* represents the most common pathogen to be identified in chronic 59 wounds, with methicillin-resistant S. aureus (MRSA) accounting for upward of 20% to 50% of cases 60 (Price, 2010). MRSA colonies often develop at the interface between synthetic prostheses and 61 biological tissues, particularly during surgery and post-surgery course. In addition, MRSA colonization 62 or infection of wounds can result in MRSA bacteremia, which is associated with a 30-day mortality of 63 64 about 28% to 38% patients (Gurusamy et al., 2013).

The main goal of chronic wound treatment is to decrease the injuring-associated microbial load, thus allowing wound healing processes to take place. However, conventional systemic delivery of antibiotics not only entails poor penetration into ischemic and necrotic tissues, but can also cause systemic toxicity with associated renal and liver complications, resulting in forced hospitalization for further monitoring and advanced treatment. On the contrary, topically applied antimicrobials have proven effective in decreasing bacterial levels in granulating wounds (Diehr et al., 2008). Therefore, alternative local delivery of antimicrobials - either by topical administration or through novel delivery
 devices - may enable to keep high local antibiotic concentrations for prolonged release times without
 reaching systemic toxicity (Zilberman et al., 2008).

A promising approach to develop a topical therapy for microbial infection in skin and soft tissues 74 would employ biocompatible nanomaterials and drug nanocarriers. Indeed, nanotechnology represents 75 an emerging field to be exploited for antibiotic drug delivery. Thanks to their physical and chemical 76 77 properties (small size, high surface-to-volume ratio and suitable surface modification) nano-sized materials may be used as drug carriers to trespass several physiological barriers and to reach biological 78 79 targets. The coupling of nanocarriers with anti-infectious agents makes it likely to increase drug 80 concentrations and drug penetration at the site of infection. As a result, it might not only improve the therapeutic index but also reduce some issues associated with nonspecific cytotoxicity and antibiotic 81 82 resistance (Sharma et al., 2012).

Vancomycin hydrochloride, being effective against many Gram-positive bacteria that are unresponsive 83 to common antibiotics, represents the gold standard against MRSA infections (Kullar et al., 2016). 84 However, Vm is poorly absorbed from the gastrointestinal tract with a low oral bioavailabiliy. Low 85 intravenous infusion is often suggested as a feasible alternative for drug administration, but Vm 86 instability in aqueous solutions at 37°C could imply a tremendous reduction of drug effectiveness 87 88 (Mawhinney et al., 1992; Raverdy et al., 2013). Following parenteral administration, Vm displays a slow mode of action, a complex concentration-time profile, and a disappointingly low penetration in 89 tissues (Vandecasteele et al., 2012). Furthermore, systemic Vm administration can be associated with 90 91 several adverse effects (Vidal et al., 1992). On the other hand, Vm topical application - that would be much safer than systemic administration - is currently limited by several factors such as skin barrier 92 properties and poor drug permeability (Giandalia et al., 2001). Being the main goal of chronic wound 93 treatment to decrease the microbial load, allowing the healing processes to take place, new delivery 94

protocol should be devised, since conventional systemic delivery of antibiotics requires a drug concentration which is locally ineffective because of the poor penetration into ischemic and necrotic tissues, but can cause systemic toxicity and topically applied antimicrobials have proven effective in decreasing bacterial levels in granulating wounds (Diehr et al., 2007), without inducing systemic toxicity (Zilberman et al., 2008) but suffer from poor diffusion across membranes.

Intriguingly, the use of a nanocarrier may help to avoid the abovementioned drawbacks. Notably, 100 101 nanocarriers such as liposomes, microemulsions, and lipid nanoparticles have the potential to deliver drugs to the skin more efficiently than conventional topical carriers such as creams and ointments, that 102 103 are not usually recommended for applications on injured skin (Giandalia et al., 2001; Prabhu et al., 104 2012). However, the response to drug topical applications has been too weak so far, mainly due to the inability to cross the external skin barrier (stratum corneum) and reach the dermal regions where the 105 bacteria are nested. Interestingly, physical media such as ultrasound (US) are reportedly able to trigger 106 drug release at the site of infection by temporarily increasing skin permeability through sonophoresis. 107 As such, US is useful to promote drug targeting and transdermal delivery in a non-invasive manner 108 (Azagury et al., 2014; Park et al, 2012). 109

Microbubbles (MBs) (Guiot et al., 2006), nanobubbles (NBs) (Cavalli et al., 2009a; Cavalli et al., 110 2009b; Cavalli et al., 2016) and nanodroplets (NDs) (Magnetto et al., 2014; Prato et al., 2015) are 111 112 suitable carriers to be combined with such a physical trigger. They are spherical core-shell structures filled with gases such as perfluorocarbons. Particularly, oxygen-cored nanostructures can be employed 113 both for sonography (as contrast agents) (Fokong et al., 2012; Marxer et al., 2011) and for therapy (as 114 115 hypoxia- and infection-counteracting devices) (Gulino et al., 2015; Banche et al., 2015; Khadjavi et al., 2015; Basilico et al., 2015; Prato et al., 2016). In particular NBs, consisting in an outer shell of a 116 biocompatible/biodegradable polysaccharide (chitosan, dextran, or dextran sulfate) and an inner core 117 filled with an oxygen-storing fluorocarbon (perfluoropentane, PFP), have been purposely developed as 118

a new non-invasive, low-cost and multipurpose nanotechnological platform (Cavalli et al., 2009a; 119 120 Cavalli et al., 2009b; Cavalli et al., 2016). PFP is a perfluorocarbon with a boiling point of 29°C, hence liquid at room temperature. The use of PFP allows liquid droplet generation at room temperature. Then, 121 PFP in nanodroplets can be activated by an external stimulus, like US, by means of a mechanism called 122 123 acoustic droplet vaporization, causing the droplet to become a bubble. Depending on the properties of the nanostructure, NBs can be subsequently coupled with different molecules, such as drugs or genetic 124 materials, thus acting as nanocarriers (Cavalli et al., 2012; Cavalli et al., 2013; Delalande et al., 2012; 125 Yin et al., 2014). Due to their structure and their gaseous core, NBs are very responsive to US and can 126 take advantage from a number of effects related to microcavitation and microstreaming, occurring at 127 128 the liquid-membrane interface and responsible for transitory and reversible openings of the pores, thus crossing the membrane itself and delivering their content beyond the tissue (sonophoresis) or the cell 129 (sonoporation) membrane (Karshafian et al., 2009). 130

Based on these preconditions, the present work aimed at producing dextran sulfate-shelled and PFP-131 cored NBs for Vm local delivery to potentially treat skin infectious diseases. The formulation is 132 referred to as "nanobubbles" for sake of simplicity but it must be said that, prior to the application of 133 US, it would be more accurate to use the term "nanodroplets" when the core is constituted of PFP. 134 Therefore, Vm-loaded NBs (VmLNBs) were prepared and characterized for physico-chemical 135 parameters and drug release kinetics; tested for biocompatibility with human skin cells and for their 136 antibacterial properties or interactions with MRSA; and challenged for responsiveness to US, in order 137 to assess their effectiveness as Vm nanocarriers for local delivery. 138

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142 **2.1. Materials**

All materials were from Sigma-Aldrich, St Louis, MO, unless those indicated as follows. Sterile 143 plastics were from Costar, Cambridge, UK; ethanol (96%) was from Carlo Erba (Milan, Italy); soybean 144 lecithin (Epikuron 200[®]) was from Cargill (Hamburg, Germany); 1-800 Millipore system to obtain 145 ultrapure water and Amicon[®] Ultra-0.5 centrifugal filter device were from Millipore (Molsheim, 146 France); Ultra-Turrax SG215 homogenizer was from IKA (Staufen, Germany); RPMI 1640 medium 147 was from Invitrogen (Carlsbad, CA); Nanobrook 90Plus Particle Size Analyzer was from Brookhaven 148 (New York City, NY); Philips CM10 electron microscope was from Philips (Eindhoven, the 149 Netherlands); Ubbelhode capillary viscosimeter was from SCHOTT Instruments GmbH (Mainz, 150 Germany); Perkin Elmer PUMP 250B was from Perkin Elmer (Waltham, MA); Flexar UV/Vis LC 151 spectrophotometer detector was from Perkin Elmer (Waltham, MA); Agilent TC C₁₈ columns were 152 from Agilent (Santa Clara, CA); Orion Model 420A pH Meter was from Thermo Scientific (Waltham, 153 MA); Semi-Micro Osmometer K-7400 was from Knauer (Berlin, Germany); Beckman Coulter Allegra 154 64R Centrifuge was from Beckman Coulter (Brea, CA); Spectra/Por cellulose membranes were from 155 Spectrum Laboratories (Rancho Dominguez, CA); HaCaT cells were from Cell Line Service GmbH 156 (Eppelheim, Germany); cell culture RPMI 1640 and Dulbecco's modified Eagle's medium (DMEM) 157 were from Invitrogen (Carlsbad, CA); streptomycin was from Cambrex Bio Science (Vervies, 158 Belgium); humidified CO₂/air-incubator was from Thermo Fisher Scientific Inc. (Waltham, MA); 159 tryptic soy broth (TSB) and tryptic soy agar (TSA) were from Merk KgaA (Darmstadt, Germany); 160 Olympus Fluoview 200 laser scanning confocal system mounted on an inverted IX70 Olympus 161 microscope was from Olympus America Inc. (Melville, NY, USA) ; SPSS 16.0 software was from 162 SPSS Inc. (Chicago, IL). 163

164 **2.2. Development and manufacturing of formulations**

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166 2.2.1. Determination of Vm and dextran sulfate interaction ratio

Increasing concentrations (0.25, 0.5, 1.0, 2.0 mg/mL) of dextran sulfate aqueous solutions (1 mL) were added to 1 mL of Vm aqueous solution (1 mg/mL) under magnetic stirring at room temperature overnight. After equilibration, the systems were separated by centrifugation (20000 rpm, 15 minutes) using a centrifugal filter device (Amicon[®] Ultra), in order to determine the amount of unbound Vm in the filtrate phase. The drug concentration in the filtrate was determined using the HPLC method described below.

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174 2.2.2. Preparation of NB, Vm, and VmLNB formulations

NBs were formulated using PFP for the inner core and dextran sulfate for the shell. A purposely tuned 175 multi-step protocol was designed. Briefly, a pre-emulsion was obtained adding 300 µL of an ethanol 176 solution containing Epikuron[®] 200 and palmitic acid (1% w/v) to 500 µL of PFP under magnetic 177 stirring. After the addition of 4.8 mL of ultrapure water, the system was homogenized using a Ultra-178 Turrax SG215 homogenizer. To obtain the polymeric NBs, 350 µL of 1% w/v dextran sulfate 179 (molecular weight = 100 kDa) aqueous solution was added drop-wise under magnetic stirring. Blank 180 NBs obtained according to this procedure were employed as control formulations in the subsequent 181 experiments. On the other hand, to obtain VmLNBs, an extra step based on drop-wise addition of a Vm 182 aqueous solution (pH 3.5) to the so-formed NBs was performed under mild stirring. Different 183 concentrations of Vm solutions were added to prepare a series of VmLNB formulations with increasing 184 drug content (0.004, 0.01, 0.1, and 1 mg/mL). VmLNBs were then purified by dialysis to eliminate 185 unbound molecules. For selected experiments, fluorescent NBs and VmLNBs were obtained by the 186

addition of 6-coumarin (1 mg/mL) to the PFP core. Alternatively, fluorescent Vm was synthesized through reaction between fluorescein isothiocyanate (FITC) and Vm. For this purpose, an amount of FITC solution in methanol (0.2 % w/v) was added to Vm aqueous solution and incubated under stirring overnight in the dark. **Figure 1** shows a representative scheme resuming the general structure of fluorescent VmLNBs. For cell experiments, NBs were prepared in phosphate buffer saline pH 7.4 (PBS). For *in vitro* permeation studies, NBs were prepared in saline solution (NaCl 0.9% w/v).

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194 **2.2.3.** NB sterilization

Firstly, the glassware and the components were sterilized at 121 °C and 2 bar. Subsequently, all NB formulations were sterilized through UV-C exposure for 20 min. Thereafter, UV-C-treated materials were incubated with cell culture RPMI 1640 medium in a humidified CO_2/air -incubator at 37°C up to 72 h, not displaying any signs of microbial contamination when checked by optical microscopy.

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200 **2.3. Characterization of formulations**

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202 2.3.1. Characterization of NB and VmLNB formulations

The average diameter, polydispersity index and zeta potential were determined by photocorrelation spectroscopy using a particle size analyzer at a scattering angle of 90° and a temperature of 25 °C. NB suspensions were diluted in deionized filtered water before measurement. For zeta potential determination, samples of diluted NB formulations were placed in the electrophoretic cell, where an electric field of approximately 15 V/cm was applied. The morphology of formulations was evaluated by Transmission Electron Microscopy (TEM), using a Philips CM10 (Eindhoven, NL) instrument. NB and VmLNB aqueous suspensions were sprayed on Formwar-coated copper grid and air-dried before 210 observation. The viscosity of the samples was determined at 25 °C using a Ubbelhode capillary 211 viscosimeter.

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213 2.3.2. HPLC quantitative Vm determination

214 Vm quantitative determination was carried out by using an HPLC system based on a Perkin Elmer pump equipped with a spectrophotometer detector. Analyses were performed using an Agilent TC C_{18} 215 column (250 mm \times 4.6 mm, 5 µm). The mobile phase was a mixture of KH₂PO₄ 50 mM (pH 4) and 216 acetonitrile (92:8 v/v), degassed and pumped through the column with a flow rate of 1 mL/min. 217 Ultraviolet detection was set at 286 nm. The external standard method was used to calculate the drug 218 concentration. For this purpose, 1 mg of Vm was weighted, placed in a volumetric flask, and dissolved 219 in water to obtain a stock standard solution. This solution was then diluted using the mobile phase, 220 providing a series of calibration solutions, subsequently injected into the HPLC system. Linear 221 calibration curve was obtained over the concentration range of 0.5-25 µg/mL, with a regression 222 coefficient of 0.999. 223

224

225 **2.3.3.** In vitro evaluation of Vm stability

226 Vm chemical stability - either solved in aqueous solution or loaded in VmLNBs - was evaluated at 227 room temperature and at 37 °C over time. A quantitative determination of Vm concentration over time 228 was carried out using the HPLC method described above.

229

230 2.3.4. NB stability over time and after US administration

The physical stability of NBs was evaluated by morphological analysis and by size and zeta potential determination of formulation over time. Their average diameters, zeta potential values and morphology were assessed up to six months. Stability was also investigated following NB exposure to US ($f = 2.5 \pm$ 234 0.1 MHz; t = 10 min; P = 5 W). NB morphology was observed by TEM to confirm the integrity of NB 235 structure.

236

237 2.3.5. Encapsulation efficiency and loading capacity of Vm in NBs

The encapsulation efficiency of VmLNBs was determined using a centrifugal filter system. 150 μ L of VmLNB suspension were put in an Amicon[®] Ultra-0.5 centrifugal filter device and centrifuged at 15000 rpm for 30 minutes using Beckman Coulter Allegra 64R Centrifuge. The solution filtered in the bottom of the tube was quantified and after suitable dilution was analyzed by HPLC, in order to obtain the concentration of free Vm in VmLNBs suspensions. The encapsulation efficiency was calculated by subtracting the amount of free drug from the initial amount of added Vm, according to the following equation:

$$Encapsulation efficiency = \frac{(total Vm - free Vm)}{total Vm} \times 100$$

The loading capacity was determined on freeze-dried NB samples. Briefly, a weighted amount of freeze-dried VmLNBs was suspended in 10 mL of water. After sonication and centrifugation, the supernatant was diluted with mobile phase and analyzed by HPLC. The loading capacity of Vm in VmLNBs was calculated as follows:

$$Loading \ capacity = \frac{(total \ Vm - free \ Vm)}{NB \ weight} \times 100$$

250 251

252 2.4. In vitro release studies

In vitro drug release experiments were conducted in a multi-compartment rotating cell, comprising a donor chamber separated by a cellulose membrane (cut-off = 12000 Da) from a receiving compartment. One ml of VmLNB suspension at different concentrations (1, 0.1, 0.01 and 0.004 mg/mL) was placed in the donor chamber. The *in vitro* release kinetics of Vm from VmLNB was compared to a Vm
aqueous solution (1 mg/mL) as a control. The receiving phase, containing phosphate buffer 0.05 M (pH
7.4) was withdrawn at regular intervals and replaced with the same amount of fresh buffer. Quantitative
determination of Vm in the withdrawn samples was carried out by the HPLC method, as described in
the previous paragraph. Data were expressed as % of Vm released over time.

261

262 2.5. In vitro permeation study

In vitro studies were performed using a vertical diffusion Franz cell to evaluate Vm permeation 263 throughout the skin. The Franz cell consists of a donor compartment, with Vm (1 mg/mL, either free or 264 carried by VmLNBs, 1x10¹² NBs/ml) and a receiving compartment containing 0.9% w/w NaCl saline 265 solution. To simulate the *stratum corneum* properties a membrane pig ear skin was used. Skin slices 266 were isolated with a dermatome from the outer side of pig ears, obtained from a local slaughterhouse, 267 and then were frozen at -18 °C. Before starting the experiments, the skin was equilibrated in NaCl 0.9 268 % w/w saline solution, in the presence of 0.01% sodium azide to preserve the skin, at 25 °C for 30 min. 269 Then, after washing with saline solution, the skin layer was inserted between the two compartments of 270 the Franz cell, with the stratum corneum side facing towards the donor chamber. The study was carried 271 out for 24 hours and the receiving phase was withdrawn at regular times and replaced with the same 272 273 amount of fresh receiving medium. The collected samples were then analyzed by HPLC to determine the amount of Vm permeated over time. US abilities to promote Vm permeation were also investigated. 274 For this purpose, a high frequency US transducer (f = 2.5 MHz; P = 5 W; t = 10 min) was combined to 275 276 a purposely modified vertical diffusion cell. Drug permeation through pig skin after US application was monitored by HPLC analysis of the cumulative amount of antibiotic reaching the receiving phase over 277 time. 278

280 **2.6. Human biocompatibility studies**

281

282 **2.6.1.** Human keratinocyte cell cultures

HaCaT, a long-term cell line of human keratinocytes immortalized from a 62-year old Caucasian male donor (Boukamp et al., 1988), was used for the assessment of Vm and VmLNB biocompatibility. Cells were grown as adherent monolayers in DMEM medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μ g/mL streptomycin and 2 mM L-glutamine in a humidified CO₂/airincubator at 37°C. Before starting the experiments, cells were washed with PBS, detached with trypsin/ethylenediaminetetraacetic acid (0.05/0.02 % v/v), washed with fresh medium and plated at a standard density (10⁶ cells/well in 6-well plates) in 2 mL of fresh medium.

290

291 **2.6.2.** *Vm and VmLNB cytotoxicity*

The potential cytotoxic effects of VmLNBs were measured as the release of lactate dehydrogenase 292 (LDH) from HaCaT cells into the extracellular medium. Briefly, cells were incubated in DMEM 293 medium for 24 h with/without 1 mg/mL Vm, either free or carried by VmLNBs, in a humidified 294 CO₂/air-incubator at 37°C. Then, 1 mL of cell supernatants was collected and centrifuged at 12000 rpm 295 for 2 min. Cells were washed with fresh medium, detached with trypsin/ethylenediaminetetraacetic acid 296 297 (0.05/0.02 % v/v), washed with PBS, resuspended in 1 mL of TRAP (82.3 mM triethanolamine, pH 7.6), and sonicated on ice with a 10 s burst. 5 μ L of cell lysates and 50 μ L of cell supernatants were 298 diluted with TRAP and supplemented with 0.5 mM sodium pyruvate and 0.25 mM NADH (300 µL as a 299 final volume) to start the reaction. The reaction was followed measuring the absorbance at 340 nm (37 300 °C) with Synergy HT microplate reader. Both intracellular and extracellular enzyme activities were 301

expressed as µmol of oxidized NADH/min/well. Finally, cytotoxicity was calculated as the net ratio
 between extracellular and total (intracellular + extracellular) LDH activities.

304

305 **2.6.3. Human keratinocyte cell viability**

Cell viability was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide 306 (MTT) assay. HaCaT cells were incubated for 24 h with/without 1 mg/mL Vm, either free or carried by 307 VmLNBs, in a humidified CO₂/air-incubator at 37°C. Thereafter, 20 µL of 5 mg/mL MTT in PBS were 308 added to cells for 3 additional hours at 37 °C. The plates were then centrifuged, the supernatants 309 discarded and the dark blue formazan crystals dissolved using 100 μ L of lysis buffer containing 20 % 310 (w/v) sodium dodecyl sulfate, 40 % N,N-dimethylformamide (pH 4.7 in 80 % acetic acid). The plates 311 312 were then read on Synergy HT microplate reader at a test wavelength of 550 nm and at a reference 313 wavelength of 650 nm.

314

315 **2.7. Microbiological assays**

316

317 2.7.1. Determination of vancomycin antimicrobial activity against MRSA

Vm solutions were freshly prepared for each experiment. Determination of the minimum inhibitory concentration (MIC) of vancomycin was carried by the microdilution broth method according to the latest Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2012). Interpretation of the results was performed as outlined in the above mentioned CLSI guidelines (CLSI 2012).

322

323 2.7.2. In vitro antibacterial efficiency of VmLNBs against MRSA.

MRSA, isolated from human ulcerated wounds (Infermi Hospital, Biella, Italy), was cultured over night at 37°C in TSB. After incubation, bacteria were re-suspended in 100 mL of TSB, harvested by 10

min centrifugation at 4,000 rpm, diluted in TSB to 10⁴ colony-forming-unit (CFU)/mL, as confirmed 326 by colony counts on TSA, and then incubated in TSB with VmLNBs, loaded with Vm at different 327 concentrations (1, 0.1, 0.01, and 0.004 mg/mL), in sterile sampling tubes for 2, 3, 4, 6, and 24 hours at 328 37°C. Controls represented by either bacteria incubated in TSB, bacteria incubated with blank NBs or 329 bacteria incubated in the presence of free Vm at different concentrations (1, 0.1, 0.01 and 0.004 330 mg/mL), were also performed. At each incubation time, serial 10-fold dilutions in saline solution (0.9% 331 NaCl) were prepared from each sample, and 100 µL of each dilution were spread on TSA, so that the 332 number of CFU/mL could be determined. 333

334

335 2.7.3. Imaging with confocal laser scanning microscopy

MRSA bacteria were grown in TSB at 37°C in agitation until reaching the concentration of 1x10^{^9} 336 CFU/mL. Then, 1 mL aliquot of bacteria was pelleted (3000g x 10 min at 4°C), resuspended in PBS 1x 337 and incubated with 6-coumarin-labeled VmLNBs, 6-coumarin-labeled NBs, or FITC-labeled Vm at a 338 339 dilution of 1:11, as for previous experiments performed on eukaryotic cells. Each sample was placed on orbital shaker (160 rpm) in the dark at 37°C for 2h and 4h. After incubation, one drop from each 340 341 suspension was streaked on poly-L-lysine-coated microscope slides and allowed to dry. Then, bacteria 342 were stained with iodide propidium (PI) in PBS 1X and again allowed to dry. Fluorescence images were taken with an Olympus IX70 inverted laser scanning confocal microscope, and captured using 343 344 FluoView 200 software.

345

346 **2.8.** *Statistical analysis*

At least three independent experiments, each one in duplicate or triplicate, were performed for every investigational study. Numerical data are shown as means \pm SEM for inferential results or as means \pm SD for descriptive results (see Cumming et al., 2007 for an exhaustive review). Imaging data are shown as representative pictures. All data were analyzed by a one-way Analysis of Variance (ANOVA)
followed by Tukey's post-hoc test (software: SPSS 16.0 for Windows, SPSS Inc., Chicago, IL). P<0.05
were considered significant.

354 **3. Results**

355

356 **3.1. Characterization of VmLNB and control (blank NB and Vm) formulations**

Before NB production, the interaction between dextran sulfate and Vm was firstly investigated to 357 optimize Vm/dextran sulfate ratio. Results indicated that Vm was complexed at 99% by dextran sulfate 358 solution until the concentration of 0.5 mg/mL (data not shown). The Vm/dextran sulfate ratio was 359 calculated corresponding to 2:1 (w/w). Based on this preliminary information, NBs were prepared 360 according to the protocol described in the Materials and Methods section. After manufacturing, 361 VmLNB and blank NB formulations (with or without 6-coumarin in the inner core) were characterized 362 physico-chemically. Results are shown in Figure 2 and Table 1. Both VmLNBs and NBs displayed 363 spherical shapes with a core-shell structure by TEM analyses. All sizes were in the nanometer range, 364 with all formulations displaying around 300 nm as a value for average diameters. All polidispersity 365 indexes were included between 0.22 and 0.25. Zeta potentials ranged from -34 mV (NBs) to -29 mV 366 (VmLNBs). The loading of Vm in the NB structure did not significantly affect the viscosity of the 367 formulations. NBs were able to load Vm with an encapsulation efficiency of 86% and loading capacity 368 of 29%. 369

370

371 **3.2. Stability of VmLNB and control (blank NB and Vm) formulations**

NB and VmLNB formulations proved to be physically stable over time, as confirmed by long-term checking of the parameters assessed in the previous paragraph. Indeed, the obtained values did not remarkably change up to six months after the manufacturing of the formulations stored at 4 °C (data not shown). Furthermore, the chemical stability of the drug was comparatively checked between free Vm solution and VmLNB aqueous suspension either over time (up to 14 days) or at different temperatures (25°C and 37°C). As shown in **Figure 3**, the drug resulted much more stable from a 378 chemical point of view when properly incorporated in the nanocarrier (VmLNBs) than as such in 379 solution.

380

381 **3.3. Human biocompatibility**

382

The potential toxicity of Vm solution and VmLNB suspension on human skin cells was assessed by testing *in vitro* cultured HaCaT keratinocytes. Cells were incubated for 24 h alone, with 10% v/v Vm solution, or with VmLNB nanosuspensions in normoxic conditions (20% O₂). Thereafter, cytotoxicity was analyzed by LDH assay, and cell viability by MTT assay. As shown in **Figure 4**, neither Vm nor VmLNBs did show significant toxic effects and HaCaT cell viability was not significantly affected by either formulation.

389

390 **3.4.** *In vitro* drug release from VmLNBs

In vitro drug release from VmLNB nanosuspension and free Vm solution were comparatively 391 evaluated over time. As shown in Figure 5 (time course studies up to 6 h) and Table 2 (end-point data 392 up to 24 h), 1 mg/mL Vm release from VmLNBs was slow and prolonged over time, compared to free 393 drug solution diffusion. No initial burst effect was observed indicating Vm incorporation in NB shell. 394 Further information on additional incubation times and drug concentrations for VmLNBs is available in 395 Supplementary Materials (Table S3). Vm/VmLNB drug release ratios at different times (2, 3, 4, 6, and 396 24 h) were also calculated (see **Table 2**), in order to allow normalization of the results from treatment 397 398 with VmLNBs in the microbiological experiments described in the following paragraph.

399

400

402 **3.5.** *In vitro* antimicrobial activity of VmLNBs

403 According to preliminary microbiological analyses performed on the MRSA strain employed in the present experiments, 0.004 mg/mL resulted as the MIC value for Vm. Therefore, decreasing Vm 404 concentrations from 1 mg/mL (used for the studies described in the previous paragraphs) to 0.004 405 mg/mL (MIC value) were employed in a series of experiments aimed at comparatively evaluating Vm 406 (either free or carried by VmLNBs) antibacterial effectiveness against MRSA. Bacteria were incubated 407 at different times (2, 3, 4, 6, and 24 h) either alone (ctr) or with free Vm, VmLNBs, or blank NBs. The 408 initial drug concentrations (1; 0.1; 0.01; and 0.004 mg/mL) loaded on VmLNBs were the same as those 409 solved in free Vm solution. However, as emerged in the previous paragraph, drug release from 410 411 VmLNBs is significantly slower than free Vm solution diffusion. For this reason, before proceeding with the analysis of the results, all values on bacterial growth referring to Vm- and VmLNB-treated 412 samples were normalized upon time-dependent Vm/VmLNB drug release ratios shown in Table 2. 413 414 Normalized results are shown in **Figure 6**, whereas raw data are available in Supplementary Materials (Figure S2). 1 mg/mL Vm effectively inhibited bacterial growth at all times, independently from being 415 free or carried by the nanocarrier. Lower drug concentrations of free Vm solution were effective 416 against MRSA only after longer times of incubation (at least 3 h for 0.1 mg/mL and 0.01 mg/mL Vm; 417 and at least 4 h for 0.004 mg/mL Vm). Interestingly, Vm antibacterial efficacy was significantly 418 419 improved when the drug was carried by VmLNBs. Indeed, VmLNB-dependent inhibition of bacterial growth was significantly enhanced compared to free Vm solution, at all drug concentrations. 420 Additionally, compared to free Vm solution, VmLNB antibacterial effects appeared earlier, as they 421 422 were already evident after 2 h of incubation (the first time-point of the observational period) at all Vm concentrations. Blank NBs did not show any antibacterial activity. 423

424	Further analysis by confocal microscopy (Figure 7) displayed that MRSA avidly internalized free
425	fluorescent Vm already after 2 h of incubation, but not fluorescent VmLNBs. Fluorescent Vm-free NBs
426	did adhere to the bacterial cell wall without being internalized.

427 **3.6. US-triggered drug permeation**

The ability of US to promote Vm permeation through the skin was assayed by employing a purposely modified Franz cell constituted by a donor and a recipient chamber separated by a pig skin layer (see **Figure 8A** for a schematic representation of the apparatus). As shown in **Figure 8B**, the administration of US (t = 10 min; f = 2.5 MHz; P = 5 W) strongly induced VmLNBs to deliver the antibiotic drug from the donor chamber throughout the pig skin membrane into the recipient chamber up to 6 h. Furthermore, drug accumulated in the skin after US treatment reached 158 μ g/cm² after 6 hours.

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435

436

438 **4. Discussion**

439

Vm currently represents the main stay against MRSA infections (Koyama et al., 2013; Kullar et al., 440 2016). However, Vm administration raises several issues that urgently need to be faced, including its 441 marked instability, low oral bioavailability, complex concentration-time profile, low tissue penetration 442 (ranging from 10% in diabetic to 30% in normal skin and soft tissues), and several adverse effects 443 (Mawhinney et al., 1992; Raverdy et al., 2013; Vandecasteele et al., 2012; Vidal et al., 1992; Giandalia 444 et al., 2001). In the attempt to counteract these drawbacks, thus improving the effectiveness of Vm 445 delivery, some novel nanocarriers have been developed: i) Vm coupling to chitosan as an ocular drug 446 447 delivery vehicle for topical use in rabbit eyes has appeared more effective than carrier-free Vm (Khangtragool et al., 2011); ii) PEGylated liposomal Vm enhanced the effective treatment of MRSA 448 pneumonia and simultaneously reduced the nephrotoxicity risk compared with conventional and non-449 PEGylated Vm formulations (Muppidi et al., 2011); iii) Vm-loaded liposomes, stabilized with chitosan 450 modified gold nanoparticles bounded to their surface, have proven effective in inhibiting the bacterial 451 growth (Pornpattananangkul et al., 2011); and iv) Vm-containing trehalose and hydroxyethylcellulose 452 spherical matrices have been developed as new delivery systems suitable for topical applications on 453 extensive and purulent wounds (Giandalia et al., 2001). Recently, Vm-loaded polymersosomes were 454 455 developed from a novel pegylated oleic acid polymer for sustained antibiotic delivery (Omolo et al., 2017). Overall, these works represent the proof-of-principle for the feasibility of choice of nanocarriers, 456 as alternative drug delivery systems to obtain the desired drug release rates and bioavailability 457 (Kalhapure et al., 2015). However, the effectiveness of those nanocarriers was seriously undermined by 458 their poor ability to cross the *stratum corneum*, a skin barrier displaying low permeability unless proper 459 exogenous physical stimuli are provided (Azagury et al., 2014; Park et al, 2012). 460

For these reasons, the present study aimed at developing Vm nanocarriers as a new platform to be 461 effectively and safely employed for Vm topical administration to treat wound infections. To this 462 purpose, NBs with core-shell nanostructures were identified as first choice carriers due to their known 463 benefits in association with drug delivery, including small size, stability, suitability for drug loading, 464 responsiveness to external stimuli such as US, and controlled drug release abilities (Marano et al., 465 2016; Cavalli et al., 2009a; Cavalli et al., 2009b; Cavalli et al., 2016). In this study, dextran sulfate was 466 chosen as main constituent of the polysaccharidic shell as a consequence of the large amount of data 467 from the literature supporting dextran biocompatibility (Bos et al., 2005; De Groot et al., 2001). 468 Encouragingly, dextran-based hydrogels have already been employed as matrices in tissue engineering, 469 470 without showing signs of inflammation in vivo (Möller et al., 2007), and recent toxicological studies have shown that dextran, as well as the products from its mechano-chemical processing, can be 471 classified as class 4 (low-toxicity) substances (Dushkin et al., 2013). Moreover, dextran sulfate presents 472 a negative charge that can electrostatically interact with the positive charged Vm. On the other hand, 473 PFP was employed as principal constituent of the inner core, since it is the most widely used 474 fluorocarbon in oxygenating emulsions and NB formulations (Cabrales and Intaglietta, 2013, Castro 475 and Briceno, 2010). In order to load Vm, dextran sulfate-shelled/PFP-cored NBs were then 476 functionalized by exploiting the electrostatic interactions occurring between the negatively charged 477 478 sulfate groups of the shell and the protonated amino groups of the drug. The obtained VmLNBs displayed a spherical shape and a well-defined core-shell structure with a polymeric shell thickness of 479 about 40 nm, average diameters of 300 nm, viscosity of 1.25 cP, and negatively charged surfaces. Of 480 note, the observed decrease of zeta potential values of ~ 15 % for VmLNBs (around -29 mV) with 481 respect to blank NBs (around -34 mV) confirmed the occurrence of electrostatic interactions between 482 positive amino groups of the drug and negative sulfate groups of the polymer, leading to a partial 483

charge neutralization of the bubble surface and allowing a good Vm encapsulation efficiency (86%)
and loading capacity (29%) in the NB systems.

In addition, it should be noticed that since the zeta potential measures charge repulsion or attraction 486 between particles, it represents a fundamental parameter to avoid nanoparticle aggregation, with zeta 487 potentials lower than -25 mV or larger than +25 mV being generally required for physical stability of 488 colloid systems (Shah and Eldridge, 2014). The stability of VmLNB formulations was further 489 confirmed by long-term checking of their size, surface charge, and viscosity values, which did not 490 show any significant changes up to six months after manufacturing, stored at 4 °C. On the other hand, 491 drug stability was comparatively checked between free Vm solution and VmLNB suspension either 492 493 over time (up to 14 days) or at different temperatures (25°C and 37°C), revealing an increased stability for Vm when properly encapsulated in the nanocarriers. This appears as an undoubtedly advantageous 494 feature for VmLNB formulations, since they might prove useful to overcome the reported instability of 495 Vm in aqueous solutions at body temperature (Mawhinney et al., 1992, Raverdy et al., 2013). 496 Interestingly, VmLNBs displayed a slow and prolonged drug release kinetics compared to Vm aqueous 497 solution, with only 16% of the drug being released from VmLNBs after 6 h. These data support the 498 hypothesis that VmLNBs may be employed as an effective drug reservoir until reaching the target site, 499 where the antibiotic would be released upon sonication at an appropriate moment only. The features of 500 501 VmLNBs might be exploited for the design of innovative wound dressing following their inclusion in polymeric base. Indeed, NBs can be dispersed in polymer gel without changing physico-chemical 502 characteristics, as previously showed (Prato et al., 2015). Another intriguing feature of VmLNBs relies 503 504 on the reported evidence that surface charges play a pivotal role in making a nanoparticle suitable for topical treatment, since they enhance its interaction with the skin and improve its therapeutic effect on 505 inflamed cutaneous tissues, either without (Abdel-Mottaleb et al., 2012) or with concomitant US 506 treatment (Lopez et al., 2011). Although cationic nanoparticles are generally preferred for topical 507

treatment due to the anionic nature of the skin (Wu et al., 2010), some authors have shown that anionic nanoparticles can be more effective (Lee et al., 2013) and less toxic (Ryman-Rasmussen et al., 2007) than the cationic ones. These latter data appear consistent with our results through investigation by biochemical assays to assess VmLNB biocompatibility with human skin tissues. Indeed, VmLNBs did not induce any *in vitro* cytotoxic effects on HaCaT keratinocytes, a skin cell line that was originally immortalized from a 62-year old donor (Boukamp et al., 1988). This peculiar information strengthens remarkably the evidence on VmLNB safety for future topical applications.

515 VmLNB and carrier-free Vm antimicrobial activity against MRSA were comparatively investigated, 516 also analyzing Vm and NB physical interaction with the bacterial cell wall by confocal microscopy. 517 Interestingly, VmLNBs were more effective in MRSA bacterial growth inhibition then free Vm, 518 promoting enhanced and earlier antibacterial effects, although they were not internalized by bacteria, 519 opposite to free Vm. This behavior appears to be a likely consequence of time-sustained release of Vm 520 from VmLNBs.

Notably, an important issue that requires caution while evaluating the feasibility for any topical drug 521 treatment is represented by the considerably low degree of permeability of the skin, the primary 522 defense system for the body. This organ consists of several layers, including the stratum corneum, the 523 epidermis, and the dermis. In particular the stratum corneum - composed of corneocytes interspersed in 524 a laminate of compressed keratin and intercorneocyte lipid lamellae - is very poorly permeable to 525 foreign molecules and represents the main obstacle to transdermal drug delivery (Naik et al., 2000). 526 However, an ideal antibiotic drug formulation should be efficiently localized in the epidermis/dermis 527 and provide a sustained drug release over time (Prabhu et al., 2012). To allow a drug to penetrate the 528 skin, several approaches have been proposed, including skin patches, ionophoresis, chemical 529 enhancers, and US-triggered sonophoresis (Park et al., 2014). 530

Interestingly, antimicrobial properties have been reported for US, although its effectiveness strongly 531 varies depending on the targeted type of pathogen (fungi vs bacteria; cocci vs bacilli; Gram-positive vs 532 Gram-negative) (Sango et al., 2014). Furthermore, synergistic effects between US and antibiotics have 533 been reported in a series of studies: i) antibiotic treatment coupled with US irradiation resulted in 534 enhanced bactericidal activity against both Gram-positive and Gram-negative bacteria, especially for 535 aminoglycosides (Yu et al., 2012); ii) the combination of Vm and US decreased S. aureus viable counts 536 by two orders of magnitude compared to Vm alone (Ayan et al., 2008); and iii) the addition of NB-537 enhanced US to doxycycline treatment improved the drug effectiveness in eradicating intracellular 538 Chlamydia trachomatis (Ikeka-Dantsuji et al., 2011). US-dependent enhancement of antibiotic action 539 on biofilms was named as a 'bioacoustic effect'. Interestingly, Vm transfer through S. epidermidis 540 biofilms was shown to be significantly enhanced by US, with bubbles being able to increase the biofilm 541 permeability to Vm (Dong et al., 2013). 542

As discussed previously, VmLNBs can be effectively employed as an important reservoir to store the 543 drug until trespassing the *stratum corneum* of the skin and reaching the target site. In order to achieve 544 the latter goal, US was assayed for its ability to induce VmLNBs to trespass an *in vitro* cutaneous layer, 545 thereby releasing Vm throughout the skin. Notably, the skin from the pig ear is widely recognized as a 546 good model for human skin permeability, since it displays human-like histological and physiological 547 548 properties, including epidermal thickness and composition, dermal structure, lipid content and general morphology (Dick and Scott, 1992). The validity of the porcine model has been established by 549 comparing the permeability of simple marker molecules with the corresponding values across human 550 skin (Herkenne et al., 2006, Sekkat et al., 2002). Therefore, the porcine ear skin represents so far the 551 most accountable in vitro model to mimic the human skin in studies on percutaneous penetration 552 (Jacobi et al., 2007). In our experiments, US appeared essential to promote Vm release from VmLNBs 553 throughout the pig skin layers, in line with previous reports on NBs and sonophoresis. On the contrary, 554

the passive transport of free vancomycin hydrochloride was negligible, being a charged and hydrophilic molecule. The amount of Vm accumulated in the skin after US application combined with NBs was greater than MIC value.

558

559 **5. Conclusions**

In the present work, dextran sulfate-shelled and PFP-filled NBs were developed for Vm delivery. VLNBs proved to be effective in MRSA bacterial killing without showing toxic effects on human keratinocytes. The combination of NBs and US enhanced Vm permeation through pig skin and promoted drug skin accumulation. Based on these results, Vm topical administration through proper NB formulations might be a promising strategy for the local treatment of MRSA skin infections. The study represents the proof of concept for the future development of advanced multifunctional therapeutic systems to treat infected wounds.

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830 Figure legends

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Figure 1. Schematic structure of VmLNB formulations. Vm nanocarriers described in the present work display a core-shell structure. PFP was employed as core fluorocarbon, whereas dextran sulfate was chosen as polysaccharidic shell molecule. Vm was inserted into the outer shell throughout dextran sulfate chains. In selected experiments, VmLNBs were further functionalized by including fluorescent 6-coumarin in the inner core.

837

Figure 2. NB and VmLNB morphology. NBs and VmLNBs were checked for morphology by TEM.
Results are shown as representative images from three different preparations. Panel A. NB image by
TEM. Panel B. VmLNB image by TEM. (see also Figure S1 in Supplementary Materials for additional
images of multiple nanobubbles within the same field).

842

Figure 3. Stability of Vm and VmLNB formulations. The stability of Vm solution and VmLNB suspension was monitored up to 14 days either at room temperature (Panel A) or at 37°C (Panel B) through analysis by HPLC. Results are shown as means \pm SD from three different preparations for each formulation. Data were also analyzed for significance by ANOVA. Versus Vm solution: * *p*<0.001.

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Figure 4. Biocompatibility of Vm and VmLNBs with human keratinocytes *in vitro*. HaCaT cells (10^6 cells/2 mL DMEM medium implemented with 10% FCS) were left untreated (ctr) or treated with 200 µL of Vm solution or VmLNB suspension for 24 h in normoxia (20% O₂). Thereafter, Vm and VmLNB cytotoxicity were measured through LDH assay (Panel A), whereas cell viability was measured through MTT assay (Panel B). Results are shown as means ± SEM from three independent experiments. Data were also evaluated for significance by ANOVA. No significant differences were found among all conditions.

Figure 5. *In vitro* Vm release from Vm and VmLNB formulations. Vm release from Vm solution and VmLNB suspension was monitored up to 6 h. Results are shown as means \pm SD from three different preparations for each formulation. Data were also analyzed for significance by ANOVA. Versus Vm solution: * *p*<0.001.

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Figure 6. Antibacterial activity of Vm and VmLNBs against MRSA. MRSA were left for 2, 3, 4, 6 860 and 24 hours at 37°C alone (ctr) or incubated with 10% v/v NBs or different concentrations of Vm, 861 either free or loaded on VmLNBs (Panel A: 1 mg/mL; Panel B: 0.1 mg/mL; Panel C: 0.01 mg/mL; 862 Panel D: 0.004 mg/mL). Results are shown as means \pm SEM from three independent experiments. Data 863 on Vm- and VmLNB-treated samples were normalized upon Vm/VmLNB release ratios reported in 864 Table 2 (see also in Supplementary Materials: Table S3 for further information on percentages of drug 865 release from VmLNBs at different times/concentrations; and Figure S2 for raw data on VmLNB 866 antibacterial effects). All data were also evaluated for significance by ANOVA. Versus ctr: * p < 0.02; 867 versus Vm: ° p < 0.05. 868

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Figure 7. Drug loading on dextran sulfate-shelled NBs prevents Vm internalization by MRSA. MRSA were left alone or incubated with 10% v/v 6-coumarin-labeled VLNBs, 6-coumarin-labeled NBs, and FITC-labeled Vm for 2h at 37°C. After staining bacteria with PI, confocal fluorescent images were taken using FITC and TRITC filters. Data are shown as representative images from three independent experiments. Magnification: 100X. Red: PI. Green: 6-coumarin or FITC.

Figure 8. US-triggered sonophoresis of VmLNBs through skin membranes. US (t = 10 min; f = 2.5 MHz; P = 5 W) abilities to induce sonophoresis and Vm permeation from VmLNBs were evaluated up to 6 h by using a vertical diffusion Franz cell consisting in two chambers (donor and recipient, respectively) separated by a pig skin layer (see scheme in Panel A). Results are shown in Panel B as means \pm SD from three independent experiments. Data were also evaluated for significance by ANOVA. Versus without US: *p* < 0.001.

Formulation	Average diameter	Polydispersity	Zeta Potential	Viscosity
	± SD (nm)	index	\pm SD (mV)	(cP)
NBs	$313.4\pm~26.4$	0.24 ± 0.02	-34.5 ± 0.38	1.22
VmLNBs	304.6 ± 14.6	0.22 ± 0.03	- 28.6 ± 1.34	1.25
Fluorescent NBs	$312.8\pm\ 22.7$	0.25 ± 0.02	- 34.1 ± 1.22	1.24
Fluorescent VmLNBs	308.9 ± 22.4	0.23 ± 0.01	-29.5 ± 1.88	1.23

Table 1. Physical-chemical characterization of NBs and VmLNBs. Liquid formulations were characterized for average diameters, polydispersity index, and zeta potential by light scattering. The viscosity (cP) of NB and VmLNB suspensions was determined at 25 °C by using a Ubbelohde capillary viscosimeter. Results are shown as means \pm SD from three preparations. See also Figures 1-2 for further detail on NB and VmLNB structure and morphology.

time	% drug release from	% drug release	Vm/VmLNB
(hours)	Vm solution	from VmLNBs	drug release ratio
2	36.57	5.99	6.11
3	45.97	7.97	5.78
4	57.16	10.27	5.57
6	73.44	14.59	5.03
24	92.34	35.84	2.58

Table 2. *In vitro* **drug release from Vm solution and VmLNB suspension.** After incubation for increasing times (first column), the percentages of *in vitro* drug release from Vm solution (second column) and VmLNB suspension (third column) were measured. Then, Vm/VmLNB drug release ratios (fourth column) were calculated for each time considered. All incubation times (2, 3, 4, 6, and 24 h) were purposely chosen to further normalize the results from the experiments with MRSA (see Figure 6). Results are shown as mean values from three different preparations for each formulation.

1	Vancomycin-loaded nanobubbles: a new platform for controlled antibiotic delivery against
2	methicillin-resistant Staphylococcus aureus infections.
3	
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23 Abstract

24

Vancomycin (Vm) currently represents the gold standard against methicillin-resistant Staphylococcus 25 aureus (MRSA) infections. However, it is associated with low oral bioavailability, formulation stability 26 issues, and severe side effects upon systemic administration. These drawbacks could be overcome by 27 Vm topical administration if properly encapsulated in a nanocarrier. Intriguingly, nanobubbles (NBs) 28 are responsive to physical external stimuli such as ultrasound (US), promoting drug delivery. In this 29 work, perfluoropentane (PFP)-cored NBs were loaded with Vm by coupling to the outer dextran sulfate 30 shell. Vm-loaded NBs (VmLNBs) displayed ~300 nm sizes, anionic surfaces and good drug 31 encapsulation efficiency. In vitro, VmLNBs showed prolonged drug release kinetics, not accompanied 32 33 by cytotoxicity on human keratinocytes. Interestingly, VmLNBs were generally more effective than 34 Vm alone in MRSA killing, with VmLNB antibacterial activity being more sustained over time as a result of prolonged drug release profile. Besides, VmLNBs were not internalized by staphylococci, 35 opposite to Vm solution. Further US association promoted drug delivery from VmLNBs through an in 36 vitro model of porcine skin. Taken together, these results support the hypothesis that proper Vm 37 encapsulation in US-responsive NBs might be a promising strategy for the topical treatment of MRSA 38 wound infections. 39

40

41 Key words

42 Nanobubbles; vancomycin; methicillin-resistant *Staphylococcus aureus*; ultrasound; prolonged release.

44 **1. Introduction**

45

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Chronic wounds fail to proceed through timely regulated and interrelated processes to restore anatomical and functional integrity of the injured tissues (Lazarus et al., 1994) such as diabetic feet, bedsores, and venous ulcers (Markova et al., 2012). To date, these types of wounds are considered like a silent epidemic, affecting a large fraction of the world population and posing a major gathering threat to the public health and economy of all developed countries (Daeschlein, 2013). Hospitalized patients are at particular risk, especially those suffering from diabetes, human immunodeficiency virus or other immune disorders, as well as those undergoing chemotherapy (Payne et al., 2008).

Beyond delayed healing processes due to different factors (hypoxia, persistent inflammation, and 53 altered balances between tissue remodelling proteinases and their inhibitors), chronic wounds are often 54 worsened by microbial infections (Gurusamy et al., 2013). Among the bacteria responsible for skin 55 infection, Staphylococcus aureus represents the most common pathogen to be identified in chronic 56 wounds, with methicillin-resistant S. aureus (MRSA) accounting for upward of 20% to 50% of cases 57 (Price, 2010). MRSA colonies often develop at the interface between synthetic prostheses and 58 biological tissues, particularly during surgery and post-surgery course. In addition, MRSA colonization 59 or infection of wounds can result in MRSA bacteremia, which is associated with a 30-day mortality of 60 about 28% to 38% patients (Gurusamy et al., 2013). 61

The main goal of chronic wound treatment is to decrease the injuring-associated microbial load, thus allowing wound healing processes to take place. However, conventional systemic delivery of antibiotics not only entails poor penetration into ischemic and necrotic tissues, but can also cause systemic toxicity with associated renal and liver complications, resulting in forced hospitalization for further monitoring and advanced treatment. On the contrary, topically applied antimicrobials have proven effective in decreasing bacterial levels in granulating wounds (Diehr et al., 200<u>8</u>7). Therefore,
alternative local delivery of antimicrobials - either by topical administration or through novel delivery
devices - may enable to keep high local antibiotic concentrations for prolonged release times without
reaching systemic toxicity (Zilberman et al., 2008).

A promising approach to develop a topical therapy for microbial infection in skin and soft tissues 71 would employ biocompatible nanomaterials and drug nanocarriers. Indeed, nanotechnology represents 72 an emerging field to be exploited for antibiotic drug delivery. Thanks to their physical and chemical 73 properties (small size, high surface-to-volume ratio and suitable surface modification) nano-sized 74 materials may be used as drug carriers to trespass several physiological barriers and to reach biological 75 targets. The coupling of nanocarriers with anti-infectious agents makes it likely to increase drug 76 concentrations and drug penetration at the site of infection. As a result, it might not only improve the 77 therapeutic index but also reduce some issues associated with nonspecific cytotoxicity and antibiotic 78 resistance (Sharma et al., 2012). 79

Vancomycin hydrochloride, being effective against many Gram-positive bacteria that are unresponsive 80 to common antibiotics, represents the gold standard against MRSA infections (Kullarrant et al., 2016). 81 However, Vm is poorly absorbed from the gastrointestinal tract with a low oral bioavailabiliy. Low 82 intravenous infusion is often suggested as a feasible alternative for drug administration, but Vm 83 instability in aqueous solutions at 37°C could imply a tremendous reduction of drug effectiveness 84 (Mawhinney et al., 1992; Raverdy et al., 2013). Following parenteral administration, Vm displays a 85 slow mode of action, a complex concentration-time profile, and a disappointingly low penetration in 86 tissues (Vandecasteele et al., 2012). Furthermore, systemic Vm administration can be associated with 87 several adverse effects (Vidal et al., 1992). On the other hand, Vm topical application - that would be 88 89 much safer than systemic administration - is currently limited by several factors such as skin barrier properties and poor drug permeability (Giandalia et al., 2001). Being the main goal of chronic wound 90

treatment to decrease the microbial load, allowing the healing processes to take place, new delivery protocol should be devised, since conventional systemic delivery of antibiotics requires a drug concentration which is locally ineffective because of the poor penetration into ischemic and necrotic tissues, but can cause systemic toxicity and topically applied antimicrobials have proven effective in decreasing bacterial levels in granulating wounds (Diehr et al., 2007), without inducing systemic toxicity (Zilberman et al., 2008) but suffer from poor diffusion across membranes.

Intriguingly, the use of a nanocarrier may help to avoid the abovementioned drawbacks. Notably, 97 nanocarriers such as liposomes, microemulsions, and lipid nanoparticles have the potential to deliver 98 drugs to the skin more efficiently than conventional topical carriers such as creams and ointments, that 99 100 are not usually recommended for applications on injured skin (Giandalia et al., 2001; Prabhu et al., 101 2012). However, the response to drug topical applications has been too weak so far, mainly due to the 102 inability to cross the external skin barrier (stratum corneum) and reach the dermal regions where the 103 bacteria are nested. Interestingly, physical media such as ultrasound (US) are reportedly able to trigger drug release at the site of infection by temporarily increasing skin permeability through sonophoresis. 104 As such, US is useful to promote drug targeting and transdermal delivery in a non-invasive manner 105 (Azagury et al., 2014; Park et al, 2012). 106

Microbubbles (MBs) (Guiot et al., 2006), nanobubbles (NBs) (Cavalli et al., 2009a; Cavalli et al., 107 2009b; Cavalli et al., 2016) and nanodroplets (NDs) (Magnetto et al., 2014; Prato et al., 2015) are 108 suitable carriers to be combined with such a physical trigger. They are spherical core-shell structures 109 filled with gases such as perfluorocarbons. Particularly, oxygen-cored nanostructures can be employed 110 both for sonography (as contrast agents) (Fokong et al., 2012; Marxer et al., 2011) and for therapy (as 111 hypoxia- and infection-counteracting devices) (Gulino et al., 2015; Banche et al., 2015; Khadjavi et al., 112 113 2015; Basilico et al., 2015; Prato et al., 2016). In particular NBs, consisting in an outer shell of a biocompatible/biodegradable polysaccharide (chitosan, dextran, or dextran sulfate) and an inner core 114

115	filled with an oxygen-storing fluorocarbon (perfluoropentane, PFP), have been purposely developed as
116	a new non-invasive, low-cost and multipurpose nanotechnological platform (Cavalli et al., 2009a;
117	Cavalli et al., 2009b; Cavalli et al., 2016). PFP is a perfluorocarbon with a boiling point of 29°C, hence
118	liquid at room temperature. The use of PFP allows liquid droplet generation at room temperature. Then,
119	PFP in nanodroplets can be activated by an external stimulus, like US, by means of a mechanism called
120	acoustic droplet vaporization, causing the droplet to become a bubble. Depending on the properties of
121	the nanostructure, NBs can be subsequently coupled with different molecules, such as drugs or genetic
122	materials, thus acting as nanocarriers (Cavalli et al., 2012; Cavalli et al., 2013; Delalande et al., 2012;
123	Yin et al., 2014). Due to their structure and their gaseous core, NBs are very responsive to US and can
124	take advantage from a number of effects related to microcavitation and microstreaming, occurring at
125	the liquid-membrane interface and responsible for transitory and reversible openings of the pores, thus
126	crossing the membrane itself and delivering their content beyond the tissue (sonophoresis) or the cell
127	(sonoporation) membrane (Karshafian et al., 2009).
128	Based on these preconditions, the present work aimed at producing dextran sulfate-shelled and PFP-

129 cored NBs for Vm local delivery to potentially treat skin infectious diseases. <u>The formulation is</u> 130 referred to as "nanobubbles" for sake of simplicity but it must be said that, prior to the application of 131 US, it would be more accurate to use the term "nanodroplets" when the core is constituted of PFP. 132 Therefore, Vm-loaded NBs (VmLNBs) were prepared and characterized for physico-chemical 133 parameters and drug release kinetics; tested for biocompatibility with human skin cells and for their

- antibacterial properties or interactions with MRSA; and challenged for responsiveness to US, in order
 to assess their effectiveness as Vm nanocarriers for local delivery.
- 136

137 2. Material and methods

138

139 **2.1. Materials**

All materials were from Sigma-Aldrich, St Louis, MO, unless those indicated as follows. Sterile 140 plastics were from Costar, Cambridge, UK; ethanol (96%) was from Carlo Erba (Milan, Italy); soybean 141 lecithin (Epikuron 200[®]) was from Cargill (Hamburg, Germany); 1-800 Millipore system to obtain 142 ultrapure water and Amicon[®] Ultra-0.5 centrifugal filter device were from Millipore (Molsheim, 143 France); Ultra-Turrax SG215 homogenizer was from IKA (Staufen, Germany); RPMI 1640 medium 144 was from Invitrogen (Carlsbad, CA); Nanobrook 90Plus Particle Size Analyzer was from Brookhaven 145 (New York City, NY); Philips CM10 electron microscope was from Philips (Eindhoven, the 146 147 Netherlands); Ubbelhode capillary viscosimeter was from SCHOTT Instruments GmbH (Mainz, 148 Germany); Perkin Elmer PUMP 250B was from Perkin Elmer (Waltham, MA); Flexar UV/Vis LC spectrophotometer detector was from Perkin Elmer (Waltham, MA); Agilent TC C₁₈ columns were 149 from Agilent (Santa Clara, CA); Orion Model 420A pH Meter was from Thermo Scientific (Waltham, 150 MA); Semi-Micro Osmometer K-7400 was from Knauer (Berlin, Germany); Beckman Coulter Allegra 151 64R Centrifuge was from Beckman Coulter (Brea, CA); Spectra/Por cellulose membranes were from 152 Spectrum Laboratories (Rancho Dominguez, CA); HaCaT cells were from Cell Line Service GmbH 153 (Eppelheim, Germany); cell culture RPMI 1640 and Dulbecco's modified Eagle's medium (DMEM) 154 were from Invitrogen (Carlsbad, CA); streptomycin was from Cambrex Bio Science (Vervies, 155 Belgium); humidified CO₂/air-incubator was from Thermo Fisher Scientific Inc. (Waltham, MA); 156 tryptic soy broth (TSB) and tryptic soy agar (TSA) were from Merk KgaA (Darmstadt, Germany); 157 Olympus Fluoview 200 laser scanning confocal system mounted on an inverted IX70 Olympus 158 159 microscope was from Olympus America Inc. (Melville, NY, USA) ; SPSS 16.0 software was from SPSS Inc. (Chicago, IL). 160

162 **2.2. Development and manufacturing of formulations**

163

164 2.2.1. Determination of Vm and dextran sulfate interaction ratio

Increasing concentrations (0.25, 0.5, 1.0, 2.0 mg/mL) of dextran sulfate aqueous solutions (1 mL) were added to 1 mL of Vm <u>aqueous</u> solution (1 mg/mL) under magnetic stirring at room temperature overnight. After equilibration, the systems were separated <u>by centrifugation (20000 rpm, 15 minutes)</u> using a centrifugal filter device (Amicon[®] Ultra), in order to determine the amount of unbound Vm in the filtrate phase. The drug concentration in the filtrate was determined using the HPLC method described below.

171

172 2.2.2. Preparation of NB, Vm, and VmLNB formulations

NBs were formulated using PFP for the inner core and dextran sulfate for the shell. A purposely tuned 173 multi-step protocol was designed. Briefly, a pre-emulsion was obtained adding 300 µmL of an ethanol 174 solution containing Epikuron[®] 200 and palmitic acid (1% w/v) to 500 μ L of PFP under magnetic 175 stirring. After the addition of 4.8 mL of ultrapure water, the system was homogenized using a Ultra-176 Turrax SG215 homogenizer. To obtain the polymeric NBs, 350 µL of 1% w/v dextran sulfate 177 (molecular weight = 100 kDa) aqueous solution was added drop-wise under magnetic stirring. Blank 178 NBs obtained according to this procedure were employed as control formulations in the subsequent 179 experiments. On the other hand, to obtain VmLNBs, an extra step based on drop-wise addition of a Vm 180 aqueous solution (pH 3.5) to the so-formed NBs was performed under mild stirring. Different 181 concentrations of Vm solutions were added to prepare a series of VmLNB formulations with increasing 182 183 drug content (0.004, 0.01, 0.1, and 1 mg/mL). VmLNBs were then purified by dialysis to eliminate unbound molecules. For selected experiments, fluorescent NBs and VmLNBs were obtained by the addition of 6-coumarin (1 mg/mL) to the PFP core. Alternatively, fluorescent Vm was synthesized through reaction between fluorescein isothiocyanate (FITC) and Vm. For this purpose, an amount of FITC solution in methanol (0.2 % w/v) was added to Vm aqueous solution and incubated under stirring overnight in the dark. **Figure 1** shows a representative scheme resuming the general structure of fluorescent VmLNBs. For cell experiments, NBs were prepared in phosphate buffer saline pH 7.4 (PBS). For *in vitro* permeation studies, NBs were prepared in saline solution (NaCl 0.9% w/v).

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192 2.2.3. NB sterilization

Firstly, the glassware and the components were sterilized at 121 °C and 2 bar. Subsequently, all NB formulations were sterilized through UV-C exposure for 20 min. Thereafter, UV-C-treated materials were incubated with cell culture RPMI 1640 medium in a humidified CO₂/air-incubator at 37°C up to 72 h, not displaying any signs of microbial contamination when checked by optical microscopy.

197

198 **2.3. Characterization of formulations**

199

200 2.3.1. Characterization of NB and VmLNB formulations

The average diameter, polydispersity index and zeta potential were determined by photocorrelation spectroscopy using a particle size analyzer at a scattering angle of 90° and a temperature of 25 °C. NB suspensions were diluted in deionized filtered water before measurement. For zeta potential determination, samples of diluted NB formulations were placed in the electrophoretic cell, where an electric field of approximately 15 V/cm was applied. The morphology of formulations was evaluated by Transmission Electron Microscopy (TEM), using a Philips CM10 (Eindhoven, NL) instrument. NB and VmLNB aqueous suspensions were sprayed on Formwar-coated copper grid and air-dried before 208 observation. The viscosity of the samples was determined at 25 °C using a Ubbelhode capillary 209 viscosimeter.

210

211 2.3.2. HPLC quantitative Vm determination

Vm quantitative determination was carried out by using an HPLC system based on a Perkin Elmer 212 pump equipped with a spectrophotometer detector. Analyses were performed using an Agilent TC C_{18} 213 column (250 mm \times 4.6 mm, 5 µm). The mobile phase was a mixture of KH₂PO₄ 50 mM (pH 4) and 214 acetonitrile (92:8 v/v), degassed and pumped through the column with a flow rate of 1 mL/min. 215 Ultraviolet detection was set at 286 nm. The external standard method was used to calculate the drug 216 concentration. For this purpose, 1 mg of Vm was weighted, placed in a volumetric flask, and dissolved 217 in water to obtain a stock standard solution. This solution was then diluted using the mobile phase, 218 providing a series of calibration solutions, subsequently injected into the HPLC system. Linear 219 220 calibration curve was obtained over the concentration range of 0.5-25 µg/mL, with a regression coefficient of 0.999. 221

222

223 2.3.3. In vitro evaluation of Vm stability

224 Vm chemical stability - either solved in aqueous solution or loaded in VmLNBs - was evaluated at 225 room temperature and at 37 °C over time. A quantitative determination of Vm concentration over time 226 was carried out using the HPLC method described above.

227

228 2.3.4. NB stability over time and after US administration

The physical stability of NBs was evaluated by morphological analysis and by size and zeta potential determination of formulation over time. Their average diameters, zeta potential values and morphology were assessed up to six months. Stability was also investigated following NB exposure to US ($f = 2.5 \pm$ 0.1 MHz; t = 10 min; P = 5 W). NB morphology was observed by TEM to confirm the integrity of NB
structure.

234

235 2.3.5. Encapsulation efficiency and loading capacity of Vm in NBs

The encapsulation efficiency of VmLNBs was determined using a centrifugal filter system. 150 [umicroL of VmLNB suspension were put in an Amicon[®] Ultra-0.5 centrifugal filter device and centrifuged at 15000 rpm for 30 minutes using Beckman Coulter Allegra 64R Centrifuge. The solution filtered in the bottom of the tube was quantified and after suitable dilution was analyzed by HPLC, in order to obtain the concentration of free Vm in VmLNBs suspensions. The encapsulation efficiency was calculated by subtracting the amount of free drug from the initial amount of added Vm, according to the following equation:

Encapsulation efficiency =
$$\frac{(total Vm - free Vm)}{total Vm} \times 100$$
243

The loading capacity was determined on freeze-dried NB samples. Briefly, a weighted amount of freeze-dried VmLNBs was <u>suspendeddiluted</u> in <u>105</u> mL of water. After sonication and centrifugation, the supernatant was <u>diluted with mobile phase and</u> analyzed by HPLC. The loading capacity of Vm in VmLNBs was calculated as follows:

$$Loading \ capacity = \frac{(total \ Vm - free \ Vm)}{NB \ weight} \times 100$$

248 249

250 2.4. In vitro release studies

In vitro drug release experiments were conducted in a multi-compartment rotating cell, comprising a donor chamber separated by a cellulose membrane (cut-off = 12000 Da) from a receiving compartment. One ml of VmLNB suspension at different concentrations (1, 0.1, 0.01 and 0.004 mg/mL) was placed in the donor chamber. The *in vitro* release kinetics of Vm from VmLNB was compared to a Vm aqueous solution (1 mg/mL) as a control. The receiving phase, containing phosphate buffer 0.05 M (pH 7.4) was withdrawn at regular intervals and replaced with the same amount of fresh buffer. Quantitative determination of Vm in the withdrawn samples was carried out by the HPLC method, as described in the previous paragraph. Data were expressed as % of Vm released over time.

259

260 2.5. In vitro permeation study

In vitro studies were performed using a vertical diffusion Franz cell to evaluate Vm permeation 261 throughout the skin. The Franz cell consists of a donor compartment, with Vm (1 mg/mL, either free or 262 carried by VmLNBs, 1x10¹² NBs/ml) and a receiving compartment containing 0.9% w/w NaCl saline 263 264 solution. To simulate the *stratum corneum* properties a membrane pig ear skin was used. Skin slices were isolated with a dermatome from the outer side of pig ears, obtained from a local slaughterhouse, 265 and then were frozen at -18 °C. Before starting the experiments, the skin was equilibrated in NaCl 0.9 266 % w/w saline solution, in the presence of 0.01% sodium azide to preserve the skin, at 25 °C for 30 min. 267 Then, after washing with saline solution, the skin layer was inserted between the two compartments of 268 the Franz cell, with the stratum corneum side facing towards the donor chamber. The study was carried 269 out for 24 hours and the receiving phase was withdrawn at regular times and replaced with the same 270 amount of fresh receiving medium. The collected samples were then analyzed by HPLC to determine 271 the amount of Vm permeated over time. US abilities to promote Vm permeation were also investigated. 272 For this purpose, a high frequency US transducer (f = 2.5 MHz; P = 5 W; t = 10 min) was combined to 273 a purposely modified vertical diffusion cell. Drug permeation through pig skin after US application was 274 monitored by HPLC analysis of the cumulative amount of antibiotic reaching the receiving phase over 275 276 time.

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278 **2.6. Human biocompatibility studies**

279

280 2.6.1. Human keratinocyte cell cultures

HaCaT, a long-term cell line of human keratinocytes immortalized from a 62-year old Caucasian male donor (Boukamp et al., 1988), was used for the assessment of Vm and VmLNB biocompatibility. Cells were grown as adherent monolayers in DMEM medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μ g/mL streptomycin and 2 mM L-glutamine in a humidified CO₂/airincubator at 37°C. Before starting the experiments, cells were washed with PBS, detached with trypsin/ethylenediaminetetraacetic acid (0.05/0.02 % v/v), washed with fresh medium and plated at a standard density (10⁶ cells/well in 6-well plates) in 2 mL of fresh medium.

288

289 2.6.2. Vm and VmLNB cytotoxicity

The potential cytotoxic effects of VmLNBs were measured as the release of lactate dehydrogenase 290 (LDH) from HaCaT cells into the extracellular medium. Briefly, cells were incubated in DMEM 291 medium for 24 h with/without 1 mg/mL Vm, either free or carried by VmLNBs, in a humidified 292 CO₂/air-incubator at 37°C. Then, 1 mL of cell supernatants was collected and centrifuged at 12000 293 rpm13000g for 2 min. Cells were washed with fresh medium, detached 294 with trypsin/ethylenediaminetetraacetic acid (0.05/0.02 % v/v), washed with PBS, resuspended in 1 mL of 295 TRAP (82.3 mM triethanolamine, pH 7.6), and sonicated on ice with a 10 s burst. 5 umicroL of cell 296 297 lysates and 50 microul L of cell supernatants were diluted with TRAP and supplemented with 0.5 mM sodium pyruvate and 0.25 mM NADH (300 umieroL as a final volume) to start the reaction. The 298 reaction was followed measuring the absorbance at 340 nm (37 °C) with Synergy HT microplate 299 reader. Both intracellular and extracellular enzyme activities were expressed as µmol of oxidized 300

301 NADH/min/well. Finally, cytotoxicity was calculated as the net ratio between extracellular and total

- 302 (intracellular + extracellular) LDH activities.
- 303

304 2.6.3. Human keratinocyte cell viability

Cell viability was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide 305 (MTT) assay. HaCaT cells were incubated for 24 h with/without 1 mg/mL Vm, either free or carried by 306 VmLNBs, in a humidified CO₂/air-incubator at 37°C. Thereafter, 20 microuL of 5 mg/mL MTT in 307 308 PBS were added to cells for 3 additional hours at 37 °C. The plates were then centrifuged, the 309 supernatants discarded and the dark blue formazan crystals dissolved using 100 µL of lysis buffer containing 20 % (w/v) sodium dodecyl sulfate, 40 % N,N-dimethylformamide (pH 4.7 in 80 % acetic 310 acid). The plates were then read on Synergy HT microplate reader at a test wavelength of 550 nm and 311 312 at a reference wavelength of 650 nm.

313

314 2.7. Microbiological assays

315

316 2.7.1. Determination of vancomycin antimicrobial activity against MRSA

Wr solutions were freshly prepared for each experiment. Determination of the minimum inhibitory concentration (MIC) of vancomycin was carried by the microdilution broth method according to the latest Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2012). Interpretation of the results was performed as outlined in the above mentioned CLSI guidelines (CLSI 2012).

321

322 2.7.2. In vitro antibacterial efficiency of VmLNBs against MRSA.

MRSA, isolated from human ulcerated wounds (Infermi Hospital, Biella, Italy), was cultured over night at 37°C in TSB. After incubation, bacteria were re-suspended in 100 mL of TSB, harvested by 10

min centrifugation at 4,000 rpm, diluted in TSB to 10⁴ colony-forming-unit (CFU)/mL, as confirmed 325 by colony counts on TSA, and then incubated in TSB with VmLNBs, loaded with Vm at different 326 concentrations (1, 0.1, 0.01, and 0.004 mg/mL), in sterile sampling tubes for 2, 3, 4, 6, and 24 hours at 327 37°C. Controls represented by either bacteria incubated in TSB, bacteria incubated with blank NBs or 328 bacteria incubated in the presence of free Vm at different concentrations (1, 0.1, 0.01 and 0.004 329 mg/mL), were also performed. At each incubation time, serial 10-fold dilutions in saline solution (0.9% 330 NaCl) were prepared from each sample, and 100 µmieroL of each dilution were spread on TSA, so that 331 332 the number of CFU/mL could be determined.

333

334 2.7.3. Imaging with confocal laser scanning microscopy

MRSA bacteria S. aureus strain werewas grown in TSB at 37°C in agitation until reaching the 335 concentration of 1x10^{^9} CFU/mL. Then, 1 mL aliquot of bacteria was pelleted (3000g x 10 min at 336 4°C), resuspended in PBS 1x and incubated with 6-coumarin-labeled VmLNBs, 6-coumarin-labeled 337 338 NBs, or FITC-labeled Vm at a dilution of 1:11, as for previous experiments performed on eukaryotic cells. Each sample was placed on orbital shaker (160 rpm) in the dark at 37°C for 2h and 4h. After 339 340 incubation, one drop from each suspension was streaked on poly-L-lysine-coated microscope slides and allowed to dry. Then, bacteria were stained with iodide propidium (PI) in PBS 1X and again allowed to 341 342 dry. Fluorescence images were taken with an Olympus IX70 inverted laser scanning confocal 343 microscope, and captured using FluoView 200 software.

344

345 **2.8.** Statistical analysis

At least three independent experiments, each one in duplicate or triplicate, were performed for every investigational study. Numerical data are shown as means \pm SEM for inferential results or as means \pm SD for descriptive results (see Cumming et al., 2007 for an exhaustive review). Imaging data are shown

- 349 as representative pictures. All data were analyzed by a one-way Analysis of Variance (ANOVA)
- followed by Tukey's post-hoc test (software: SPSS 16.0 for Windows, SPSS Inc., Chicago, IL). P<0.05
- 351 were considered significant.

353 **3. Results**

354

355 3.1. Characterization of VmLNB and control (blank NB and Vm) formulations

Before NB production, the interaction between dextran sulfate and Vm was firstly investigated to 356 optimize Vm/dextran sulfate ratio. Results indicated that Vm was complexed at 99% by dextran sulfate 357 solution until the concentration of 0.5 mg/mL4 (data not shown). The Vm/dextran sulfate ratio was 358 calculated corresponding to 2:1 (w/w). Based on this preliminary information, NBs were prepared 359 according to the protocol described in the Materials and Methods section. After manufacturing, 360 VmLNB and blank NB formulations (with or without 6-coumarin in the inner core) were characterized 361 physico-chemically. Results are shown in Figure 2 and Tables 1-2. Both VmLNBs and NBs displayed 362 spherical shapes with a core-shell structure by TEM analyses. All sizes were in the nanometer range, 363 with all formulations displaying around 300 nm as a value for average diameters. All polidispersity 364 indexes were included between 0.22 and 0.25. Zeta potentials ranged from -34 mV (NBs) to -29 mV 365 (VmLNBs). The loading of Vm in the NB structure did not significantly affect the viscosity of the 366 formulations. NB viscosity (1.12 cP) was slightly increased upon binding with Vm (1.25 cP for 367 VmLNBs).-NBs were able to load Vm with an encapsulation efficiency of 86% and loading capacity of 368 29%. 369

370

371 3.2. Stability of VmLNB and control (blank NB and Vm) formulations

NB and VmLNB formulations proved to be physically stable over time, as confirmed by long-term checking of the parameters assessed in the previous paragraph. Indeed, the obtained values did not remarkably change up to six months after the manufacturing of the formulations stored at 4 °C (data not shown). Furthermore, the chemical stability of the drug was comparatively checked between free Vm solution and VmLNB aqueous suspension either over time (up to 14 days) or at different temperatures (25°C and 37°C). As shown in Figure 3, the drug Vm always-resulted much more stable
 from a chemical point of view when properly incorporated in the nanocarriers (VmLNBs) than as such
 in solution.alone.

380

381 **3.3. Human biocompatibility**

382

The potential toxicity of Vm solution and VmLNB suspension on human skin cells was assessed by testing *in vitro* cultured HaCaT keratinocytes. Cells were incubated for 24 h alone, with 10% v/v Vm solution, or with VmLNB nanosuspensions in normoxic conditions (20% O₂). Thereafter, cytotoxicity was analyzed by LDH assay, and cell viability by MTT assay. As shown in **Figure 4**, neither Vm nor VmLNBs did show significant toxic effects and HaCaT cell viability was not significantly affected by either formulation.

389

390 3.4. In vitro drug release from VmLNBs

In vitro drug release from VmLNB nanosuspension and free Vm solution were comparatively 391 evaluated over time. As shown in Figure 5 (time course studies up to 6 h) and Table 23 (end-point data 392 up to 24 h), 1 mg/mL Vm release from VmLNBs was slow and prolonged over time, compared to free 393 drug solution diffusion. No initial burst effect was observed indicating Vm incorporation in NB shell. 394 Further information on additional incubation times and drug concentrations for VmLNBs is available in 395 Supplementary Materials (Table S₃). Vm/VmLNB drug release ratios at different times (2, 3, 4, 6, 396 and 24 h) were also calculated (see Table 23), in order to allow normalization of the results from 397 treatment with VmLNBs in the microbiological experiments described in the following paragraph. 398

399

400 3.5. In vitro antimicrobial activity of VmLNBs

According to preliminary microbiological analyses performed on the MRSA strain employed in the 401 402 present experiments, 0.004 mg/mL resulted as the MIC value for Vm. Therefore, decreasing Vm concentrations from 1 mg/mL (used for the studies described in the previous paragraphs) to 0.004 403 mg/mL (MIC value) were employed in a series of experiments aimed at comparatively evaluating Vm 404 (either free or carried by VmLNBs) antibacterial effectiveness against MRSA. Bacteria were incubated 405 at different times (2, 3, 4, 6, and 24 h) either alone (ctr) or with free Vm, VmLNBs, or blank NBs. The 406 initial drug concentrations (1; 0.1; 0.01; and 0.004 mg/mL) loaded on VmLNBs were the same as those 407 solved in free Vm solution. However, as emerged in the previous paragraph, drug release from 408 VmLNBs is significantly slower than free Vm solution diffusion. For this reason, before proceeding 409 with the analysis of the results, all values on bacterial growth referring to Vm- and VmLNB-treated 410 samples were normalized upon time-dependent Vm/VmLNB drug release ratios shown in Table 23. 411 Normalized results are shown in Figure 6, whereas raw data are available in Supplementary Materials 412 (Figure S24). 1 mg/mL Vm effectively inhibited bacterial growth at all times, independently from 413 being free or carried by the nanocarrier. Lower drug concentrations of free Vm solution were effective 414 against MRSA only after longer times of incubation (at least 3 h for 0.1 mg/mL and 0.01 mg/mL Vm; 415 and at least 4 h for 0.004 mg/mL Vm). Interestingly, Vm antibacterial efficacy was significantly 416 improved when the drug was carried by VmLNBs. Indeed, VmLNB-dependent inhibition of bacterial 417 growth was significantly enhanced compared to free Vm solution, at all drug concentrations. 418 Additionally, compared to free Vm solution, VmLNB antibacterial effects appeared earlier, as they 419 were already evident after 2 h of incubation (the first time-point of the observational period) at all Vm 420 concentrations. Blank NBs did not show any antibacterial activity. 421

Further analysis by confocal microscopy (Figure 7) displayed that MRSA avidly internalized free
fluorescent Vm already after 2 h of incubation, but not fluorescent VmLNBs. Fluorescent Vm-free NBs
did adhere to the bacterial cell wall without being internalized.

3.6. US-triggered drug permeation

426	The ability of US to promote Vm permeation through the skin was assayed by employing a purposely
427	modified Franz cell constituted by a donor and a recipient chamber separated by a pig skin layer (see
428	Figure 8A for a schematic representation of the apparatus). As shown in Figure 8B, the administration
429	of US (t = 10 min; f = 2.5 MHz; P = 5 W) strongly induced VmLNBs to deliver the antibiotic drug
430	from the donor chamber throughout the pig skin membrane into the recipient chamber up to 6 h.
431	Furthermore, drug accumulated in the skin after US treatment reached 158 μ g/cm ² after 6 hours.
Vm currently represents the main stay against MRSA infections (Koyama et al., 2013; Kullarrant et 438 al., 2016). However, Vm administration raises several issues that urgently need to be faced, including 439 its marked instability, low oral bioavailability, complex concentration-time profile, low tissue 440 penetration (ranging from 10% in diabetic to 30% in normal skin and soft tissues), and several adverse 441 effects (Mawhinney et al., 1992; Raverdy et al., 2013; Vandecasteele et al., 2012; Vidal et al., 1992; 442 Giandalia et al., 2001). In the attempt to counteract these drawbacks, thus improving the effectiveness 443 of Vm delivery, some novel nanocarriers have been developed: i) Vm coupling to chitosan as an ocular 444 drug delivery vehicle for topical use in rabbit eyes has appeared more effective than carrier-free Vm 445 (Khangtragool et al., 2011); ii) PEGylated liposomal Vm enhanced the effective treatment of MRSA 446 pneumonia and simultaneously reduced the nephrotoxicity risk compared with conventional and non-447 PEGylated Vm formulations (Muppidi et al., 2011); iii) Vm-loaded liposomes, stabilized with chitosan 448 modified gold nanoparticles bounded to their surface, have proven effective in inhibiting the bacterial 449 growth (Pornpattananangkul et al., 2011); and iv) Vm-containing trehalose and hydroxyethylcellulose 450 spherical matrices have been developed as new delivery systems suitable for topical applications on 451 extensive and purulent wounds (Giandalia et al., 2001). Recently, Vm-loaded polymersosomes were 452 developed from a novel pegylated oleic acid polymer for sustained antibiotic delivery (Omolo et al., 453 2017). Overall, these works represent the proof-of-principle for the feasibility of choice of nanocarriers, 454 as alternative drug delivery systems to obtain the desired drug release rates and bioavailability 455 (Kalhapure et al., 2015). However, the effectiveness of those nanocarriers was seriously undermined by 456 their poor ability to cross the stratum corneum, a skin barrier displaying low permeability unless proper 457 458 exogenous physical stimuli are provided (Azagury et al., 2014; Park et al, 2012).

For these reasons, the present study aimed at developing Vm nanocarriers as a new platform to be 459 effectively and safely employed for Vm topical administration to treat wound infections. To this 460 purpose, NBs with core-shell nanostructures were identified as first choice carriers due to their known 461 benefits in association with drug delivery, including small size, stability, suitability for drug loading, 462 responsiveness to external stimuli such as US, and controlled drug release abilities (Marano et al., 463 2016; Cavalli et al., 2009a; Cavalli et al., 2009b; Cavalli et al., 2016). In this study, dextran sulfate was 464 465 chosen as main constituent of the polysaccharidic shell as a consequence of the large amount of data from the literature supporting dextran biocompatibility (Bos et al., 2005; De Groot et al., 2001). 466 Encouragingly, dextran-based hydrogels have already been employed as matrices in tissue engineering, 467 without showing signs of inflammation in vivo (Möeller et al., 2007), and recent toxicological studies 468 469 have shown that dextran, as well as the products from its mechano-chemical processing, can be classified as class 4 (low-toxicity) substances (Dushkin et al., 2013). Moreover, dextran sulfate presents 470 a negative charge that can electrostatically interact with the positive charged Vm. On the other hand, 471 PFP was employed as principal constituent of the inner core, since it is the most widely used 472 fluorocarbon in oxygenating emulsions and NB formulations (Cabrales and Intaglietta, 2013, Castro 473 and Briceno, 2010). In order to load Vm, dextran sulfate-shelled/PFP-cored NBs were then 474 functionalized by exploiting the electrostatic interactions occurring between the negatively charged 475 sulfate groups of the shell and the protonated amino groups of the drug. The obtained VmLNBs 476 displayed a spherical shape and a well-defined core-shell structure with a polymeric shell thickness of 477 about 40 nm, average diameters of 300 nm, viscosity of 1.25 cP, and negatively charged surfaces. Of 478 note, the observed decrease of zeta potential values of ~ 15 % for VmLNBs (around -29 mV) with 479 respect to blank NBs (around -34 mV) confirmed the occurrence of electrostatic interactions between 480 481 positive amino groups of the drug and negative sulfate groups of the polymer, leading to a partial

charge neutralization of the bubble surface and allowing a good Vm encapsulation efficiency (86%)
and loading capacity (29%) in the NB systems.

In addition, it should be noticed that since the zeta potential measures charge repulsion or attraction 484 between particles, it represents a fundamental parameter to avoid nanoparticle aggregation, with zeta 485 potentials lower than -25 mV or larger than +25 mV being generally required for physical stability of 486 colloid systems (Shah and Eldridge, 2014). The stability of VmLNB formulations was further 487 488 confirmed by long-term checking of their size, surface charge, and viscosity values, which did not show any significant changes up to six months after manufacturing, stored at 4 °C. On the other hand, 489 drug stability was comparatively checked between free Vm solution and VmLNB suspension either 490 over time (up to 14 days) or at different temperatures (25°C and 37°C), revealing an increased stability 491 for Vm when properly encapsulated in the nanocarriers. This appears as an undoubtedly advantageous 492 feature for VmLNB formulations, since they might prove useful to overcome the reported instability of 493 Vm in aqueous solutions at body temperature (Mawhinney et al., 1992, Raverdy V-et al., 2013). 494 Interestingly, VmLNBs displayed a slow and prolonged drug release kinetics compared to Vm aqueous 495 solution, with only 16% of the drug being released from VmLNBs after 6 h. These data support the 496 hypothesis that VmLNBs may be employed as an effective drug reservoir until reaching the target site, 497 where the antibiotic would be released upon sonication at an appropriate moment only. The features of 498 VmLNBs might be exploited for the design of innovative wound dressing following their inclusion in 499 polymeric base. Indeed, NBs can be dispersed in polymer gel without changing physico-chemical 500 characteristics, as previously showed (Prato et al., 2015). Another intriguing feature of VmLNBs relies 501 on the reported evidence that surface charges play a pivotal role in making a nanoparticle suitable for 502 topical treatment, since they enhance its interaction with the skin and improve its therapeutic effect on 503 504 inflamed cutaneous tissues, either without (Abdel-Mottaleb et al., 2012) or with concomitant US treatment (Lopez et al., 2011). Although cationic nanoparticles are generally preferred for topical 505

treatment due to the anionic nature of the skin (Wu et al., 2010), some authors have shown that anionic nanoparticles can be more effective (Lee et al., 2013) and less toxic (Ryman-Rasmussen et al., 2007) than the cationic ones. These latter data appear consistent with our results through investigation by biochemical assays to assess VmLNB biocompatibility with human skin tissues. Indeed, VmLNBs did not induce any *in vitro* cytotoxic effects on HaCaT keratinocytes, a skin cell line that was originally immortalized from a 62-year old donor (Boukamp et al., 1988). This peculiar information strengthens remarkably the evidence on VmLNB safety for future topical applications.

513 VmLNB and carrier-free Vm antimicrobial activity against MRSA were comparatively investigated, 514 also analyzing Vm and NB physical interaction with the bacterial cell wall by confocal microscopy. 515 Interestingly, VmLNBs were more effective in MRSA bacterial growth inhibition then free Vm, 516 promoting enhanced and earlier antibacterial effects, although they were not internalized by bacteria, 517 opposite to free Vm. This behavior appears to be a likely consequence of time-sustained release of Vm 518 from VmLNBs.

Notably, an important issue that requires caution while evaluating the feasibility for any topical drug 519 treatment is represented by the considerably low degree of permeability of the skin, the primary 520 defense system for the body. This organ consists of several layers, including the stratum corneum, the 521 epidermis, and the dermis. In particular the stratum corneum - composed of corneocytes interspersed in 522 a laminate of compressed keratin and intercorneocyte lipid lamellae - is very poorly permeable to 523 foreign molecules and represents the main obstacle to transdermal drug delivery (Naik et al., 2000). 524 However, an ideal antibiotic drug formulation should be efficiently localized in the epidermis/dermis 525 and provide a sustained drug release over time (Prabhu et al., 2012). To allow a drug to penetrate the 526 skin, several approaches have been proposed, including skin patches, ionophoresis, chemical 527 528 enhancers, and US-triggered sonophoresis (Park et al., 2014).

Interestingly, antimicrobial properties have been reported for US, although its effectiveness strongly 529 varies depending on the targeted type of pathogen (fungi vs bacteria; cocci vs bacilli; Gram-positive vs 530 Gram-negative) (Sango et al., 2014). Furthermore, synergistic effects between US and antibiotics have 531 been reported in a series of studies: i) antibiotic treatment coupled with US irradiation resulted in 532 enhanced bactericidal activity against both Gram-positive and Gram-negative bacteria, especially for 533 aminoglycosides (Yu et al., 2012); ii) the combination of Vm and US decreased S. aureus viable counts 534 by two orders of magnitude compared to Vm alone (Ayan et al., 2008); and iii) the addition of NB-535 enhanced US to doxycycline treatment improved the drug effectiveness in eradicating intracellular 536 Chlamydia trachomatis (Ikeka-Dantsuji et al., 2011). US-dependent enhancement of antibiotic action 537 on biofilms was named as a 'bioacoustic effect'. Interestingly, Vm transfer through S. epidermidis 538 biofilms was shown to be significantly enhanced by US, with bubbles being able to increase the biofilm 539 permeability to Vm (Dong Y. et al., 2013). 540

As discussed previously, VmLNBs can be effectively employed as an important reservoir to store the 541 drug until trespassing the stratum corneum of the skin and reaching the target site. In order to achieve 542 the latter goal, US was assayed for its ability to induce VmLNBs to trespass an in vitro cutaneous layer, 543 thereby releasing Vm throughout the skin. Notably, the skin from the pig ear is widely recognized as a 544 good model for human skin permeability, since it displays human-like histological and physiological 545 properties, including epidermal thickness and composition, dermal structure, lipid content and general 546 morphology (Dick and Scottet al., 1992). The validity of the porcine model has been established by 547 comparing the permeability of simple marker molecules with the corresponding values across human 548 skin (Herkenne et al., 2006, Sekkat et al., 2002). Therefore, the porcine ear skin represents so far the 549 most accountable in vitro model to mimic the human skin in studies on percutaneous penetration 550 551 (Jacobi et al., 2007). In our experiments, US appeared essential to promote Vm release from VmLNBs throughout the pig skin layers, in line with previous reports on NBs and sonophoresis. On the contrary, 552

the passive transport of free vancomycin hydrochloride was negligible, being a charged and hydrophilic molecule. The amount of Vm accumulated in the skin after US application combined with NBs was greater than MIC value.

556

557 5. Conclusions

In the present work, dextran sulfate-shelled and <u>PFPperfluoropentane</u>-filled NBs were developed for Vm delivery. VLNBs proved to be effective in MRSA bacterial killing without showing toxic effects on human keratinocytes. The combination of NBs and US enhanced Vm permeation through pig skin and promoted drug skin accumulation. Based on these results, Vm topical administration through proper NB formulations might be a promising strategy for the local treatment of MRSA skin infections. The study represents the proof of concept for the future development of advanced multifunctional therapeutic systems to treat infected wounds.

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75	Refere	nces
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832 Figure legends

833

Figure 1. Schematic structure of VmLNB formulations. Vm nanocarriers described in the present work display a core-shell structure. PFP was employed as core fluorocarbon, whereas dextran sulfate was chosen as polysaccharidic shell molecule. Vm was inserted into the outer shell throughout dextran sulfate chains. In selected experiments, VmLNBs were further functionalized by including fluorescent 6-coumarin in the inner core.

839

Figure 2. NB and VmLNB morphology. NBs and VmLNBs were checked for morphology by TEM.
Results are shown as representative images from three different preparations. Panel A. NB image by
TEM. Panel B. VmLNB image by TEM. (see also Figure S1 in Supplementary Materials for additional
images of multiple nanobubbles within the same field).

844

Figure 3. Stability of Vm and VmLNB formulations. The stability of Vm solution and VmLNB suspension was monitored up to 14 days either at room temperature (Panel A) or at 37°C (Panel B) through analysis by HPLC. Results are shown as means \pm SD from three different preparations for each formulation. Data were also analyzed for significance by ANOVA. Versus Vm solution: * *p*<0.001.

849

Figure 4. Biocompatibility of Vm and VmLNBs with human keratinocytes *in vitro*. HaCaT cells (10^6 cells/2 mL DMEM medium implemented with 10% FCS) were left untreated (ctr) or treated with 200 microµL of Vm solution or VmLNB suspension for 24 h in normoxia (20% O₂). Thereafter, Vm and VmLNB cytotoxicity were measured through LDH assay (Panel A), whereas cell viability was measured through MTT assay (Panel B). Results are shown as means ± SEM from three independent experiments. Data were also evaluated for significance by ANOVA. No significant differences were
found among all conditions.

Figure 5. In vitro Vm release from Vm and VmLNB formulations. Vm release from Vm solution and VmLNB suspension was monitored up to 6 h. Results are shown as means \pm SD from three different preparations for each formulation. Data were also analyzed for significance by ANOVA. Versus Vm solution: * p < 0.001.

861

Figure 6. Antibacterial activity of Vm and VmLNBs against MRSA. MRSA were left for 2, 3, 4, 6 862 and 24 hours at 37°C alone (ctr) or incubated with 10% v/v NBs or different concentrations of Vm, 863 either free or loaded on VmLNBs (Panel A: 1 mg/mL; Panel B: 0.1 mg/mL; Panel C: 0.01 mg/mL; 864 Panel D: 0.004 mg/mL). Results are shown as means \pm SEM from three independent experiments. Data 865 on Vm- and VmLNB-treated samples were normalized upon Vm/VmLNB release ratios reported in 866 867 Table 23 (see also in Supplementary Materials: Table 334 for further information on percentages of drug release from VmLNBs at different times/concentrations; and Figure S24 for raw data on VmLNB 868 antibacterial effects). All data were also evaluated for significance by ANOVA. Versus ctr: * p < 0.02; 869 versus Vm: ° p < 0.05. 870

871

Figure 7. Drug loading on dextran sulfate-shelled NBs prevents Vm internalization by MRSA.

MRSA were left alone or incubated with 10% v/v 6-coumarin-labeled VLNBs, 6-coumarin-labeled NBs, and FITC-labeled Vm for 2h at 37°C. After staining bacteria with PI, confocal fluorescent images were taken using FITC and TRITC filters. Data are shown as representative images from three independent experiments. Magnification: 100X. Red: PI. Green: 6-coumarin or FITC.

Figure 8. US-triggered sonophoresis of VmLNBs through skin membranes. US (t = 10 min; f = 2.5 MHz; P = 5 W) abilities to induce sonophoresis and Vm permeation from VmLNBs were evaluated up to 6 h by using a vertical diffusion Franz cell consisting in two chambers (donor and recipient, respectively) separated by a pig skin layer (see scheme in Panel A). Results are shown in Panel B as means \pm SD from three independent experiments. Data were also evaluated for significance by ANOVA. Versus without US: *p* < 0.001.

884 Tables and legends

885

Formulation	Average diameter	Polydispersity	Zeta Potential	<u>Viscosity</u>
	± SD (nm)	index	\pm SD (mV)	<u>(cP)</u>
NBs	313.4 ± 26.4	0.24 ± 0.02	-34.5 ± 0.38	<u>1.22</u>
VmLNBs	304.6 ± 14.6	0.22 ± 0.03	-28.6 ± 1.34	<u>1.25</u>
Fluorescent NBs	$312.8\pm\ 22.7$	0.25 ± 0.02	- 34.1 ± 1.22	<u>1.24</u>
Fluorescent VmLNBs	308.9 ± 22.4	0.23 ± 0.01	-29.5 ± 1.88	<u>1.23</u>

886

Table 1. Physical-chemical characterization of NBbs and VmLNBs. Liquid formulations were
characterized for average diameters, polydispersity index, and zeta potential by light scattering. The
viscosity (cP) of NB and VmLNB suspensions was determined at 25 °C by using a Ubbelohde capillary
viscosimeter. Results are shown as means ± SD from three preparations. See also Figures 1-2 for
further detail on NB and VmLNB structure and morphology.

892

Sample solutionViscosity (cP)Vm0.98NBs1.12

VmLNBs 1.25

893

894 Table 2. Viscosity of Vm solution, NB, and VmLNB suspensions. The viscosity (cP) of NB and
 895 VmLNB suspensions as well as free Vm solution was determined at 25 °C by using a Ubbelohde
 896 capillary viscosimeter. The results are reported in the table.

897

Formatted Table

time	% drug release from	% drug release	Vm/VmLNB
(hours)	Vm solution	from VmLNBs	drug release ratio
2	36.57	5.99	6.11
3	45.97	7.97	5.78
4	57.16	10.27	5.57
6	73.44	14.59	5.03
24	92.34	35.84	2.58

Table 23. *In vitro* **drug release from Vm solution and VmLNB suspension.** After incubation for increasing times (first column), the percentages of *in vitro* drug release from Vm solution (second column) and VmLNB suspension (third column) were measured. Then, Vm/VmLNB drug release ratios (fourth column) were calculated for each time considered. All incubation times (2, 3, 4, 6, and 24 h) were purposely chosen to further normalize the results from the experiments with MRSA (see Figure 6). Results are shown as mean values from three different preparations for each formulation.

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