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

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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. Vm-loaded 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 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.

1. Introduction

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 and Mostow, 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 altered balances between tissue remodelling proteinases and their inhibitors), chronic wounds are often worsened by microbial infections (Gurusamy et al., 2013). Among the bacteria responsible for skin infection, *Staphylococcus aureus* represents the most common pathogen to be identified in chronic wounds, with methicillin-resistant *S. aureus* (MRSA) accounting for upward of 20% to 50% of cases (Price, 2010). MRSA colonies often develop at the interface between synthetic prostheses and biological tissues, particularly during surgery and

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post-surgery course. In addition, MRSA colonization or infection of wounds can result in MRSA bacteremia, which is associated with a 30-day mortality of 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 and Elsner, 2008).

A promising approach to develop a topical therapy for microbial infection in skin and soft tissues would employ biocompatible nanomaterials and drug nanocarriers. Indeed, nanotechnology represents an emerging field to be exploited for antibiotic drug delivery. Thanks to their physical and chemical 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 targets. The coupling of nanocarriers with anti-infectious agents makes it likely to increase drug 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 resistance (Sharma et al., 2012).

Vancomycin hydrochloride, being effective against many Gram-positive bacteria that are unresponsive to common antibiotics, represents the gold standard against MRSA infections (Kullar et al., 2016). However, Vm is poorly absorbed from the gastrointestinal tract with a low oral bioavailabiliy. Low intravenous infusion is often suggested as a feasible alternative for drug administration, but Vm instability in aqueous solutions at 37 °C could imply a tremendous reduction of drug effectiveness (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 tissues (Vandecasteele et al., 2012). Furthermore, systemic Vm administration can be associated with 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 properties and poor drug permeability (Giandalia et al., 2001). Being the main goal of chronic wound 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., 2008), without inducing systemic toxicity (Zilberman and Elsner, 2008) but suffer from poor diffusion across membranes.

Intriguingly, the use of a nanocarrier may help to avoid the abovementioned drawbacks. Notably, 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 are not usually recommended for applications on injured skin (Giandalia et al., 2001; Prabhu et al., 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 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. As such, US is useful to promote drug targeting and transdermal delivery in a non-invasive manner (Azagury et al., 2014; Park et al., 2012).

Microbubbles (MBs) (Guiot et al., 2006), nanobubbles (NBs) (Cavalli et al., 2009a, 2009b, 2016) and nanodroplets (NDs) (Magnetto et al., 2014; Prato et al., 2015) are 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 both for sonography (as contrast agents) (Fokong et al., 2012; Marxer et al., 2011) and for therapy (as 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 biocompatible/biodegradable polysaccharide (chitosan, dextran, or dextran sulfate) and an inner core filled with an oxygen-storing fluorocarbon (perfluoropentane, PFP), have been purposely developed as a new non-invasive, low-cost and multipurpose nanotechnological platform (Cavalli et al., 2009a, 2009b, 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, PFP in nanodroplets can be activated by an external stimulus, like US, by means of a mechanism called 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 materials, thus acting as nanocarriers (Cavalli et al., 2012, 2013; Delalande et al., 2012; Yin et al., 2014). Due to their structure and their gaseous core, NBs are very responsive to US and can take advantage from a number of effects related to microcavitation and microstreaming, occurring at 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 (sonoporation) membrane (Karshafian et al., 2009).

Based on these preconditions, the present work aimed at producing dextran sulfate-shelled and PFP-cored NBs for Vm local delivery to potentially treat skin infectious diseases. The formulation is referred to as “nanobubbles” for sake of simplicity but it must be said that, prior to the application of US, it would be more accurate to use the term “nanodroplets” when the core is constituted of PFP. Therefore, Vm-loaded NBs (VmLNBs) were prepared and characterized for physico-chemical 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.

2. Material and methods

2.1. Materials

All materials were from Sigma-Aldrich, St Louis, MO, unless those indicated as follows. Sterile plastics were from Costar, Cambridge, UK; ethanol (96%) was from Carlo Erba (Milan, Italy); soybean lecithin (Epikuron 200®) was from Cargill (Hamburg, Germany); 1–800 Millipore system to obtain ultrapure water and Amicon® Ultra-0.5 centrifugal filter device were from Millipore (Molsheim, France); Ultra-Turrax SG215 homogenizer was from IKA (Staufen, Germany); RPMI 1640 medium was from Invitrogen (Carlsbad, CA); Nanobrook 90Plus Particle Size Analyzer was from Brookhaven (New York City, NY); Philips CM10 electron