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Formulation of an antibacterial topical cream containing bioengineered honey that generates reactive oxygen species

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ABSTRACT

SurgihoneyRO™ (SHRO) is a bioengineered medicinal honey proven to eradicate multi-drug resistant strains of bacteria by delivering a controlled dose of reactive oxygen species (ROS). The urgent need for novel antimicrobial therapies capable of tackling pathogens that have developed resitance to existing antimicrobial medicines, such as antibiotics, makes SHRO a highly desirable biomaterial. However, its application is currently limited in the medical field due to undesirable material properties. This study aims to formulate the honey into a clinically viable topical cream whilst maintaining antimicrobial efficacy. SHRO droplets were emulsified to protect the active until activation *in-situ*. Xanthan gum (XG) and fumed silica (FS) thickener systems were explored, with both formulations able to inhibit the growth of *S. aureus in-vitro*. However, FS formulations exhibited significantly higher hydrogen peroxide release over a period of 7 days and resulted in larger zones of inhibition (42%) than XG formulations. Selection of the optimum FS formulation was made based on evaluation of the material characteristics by means of rheology and texture analysis. In place of the sticky and highly viscous initial SHRO product, desirable material characteristics for a topical product were achieved, including thixotropic shear-thinning behaviour and significantly lower cohesiveness (15.3–22.4 N) than standard SHRO formulations (79.9 N). Furthermore, the product exhibited a low contact angle on porcine skin, indicating that these formulations would spread favourably on the skin surface, demonstrate a favourable sensory perception and be retained on the skin, making for a more clinically effective product. This work is the first report of an engineered cream system to controllably deliver ROS to a wound site and demonstrate its ability of eradicating clinically relevant bacteria *in vitro*.

1. Introduction

Antimicrobial resistance (AMR) is a public health phenomenon of global concern due to its staggering potential both clinically, and economically. It is estimated that drug-resistant infections are currently responsible for 700,000 annual deaths worldwide [1]. If current trends do not change, deaths could reach 10 million by 2050, with an associated cost of $100 trillion [1]. Therefore, research into new antimicrobials and effective delivery systems is crucial to the future success of global healthcare [2,3].

Honey has featured throughout history as a treatment for a range of ailments [4] and has been explored extensively for its antimicrobial properties in recent years [5–16]. This sweet treat is rich in components with antimicrobial potential, such as polyphenols, methylgloxal (in the famed Manuka honey) and bee defensin-1 [17–20]. SurgihoneyRO™ (SHRO) is a biochemically engineered, medical grade honey that is highly potent against multidrug-resistant bacteria [16,21] and retains the established wound-healing properties of natural honey [19,22]. SHRO is organic in the sense that it contains no agricultural additives or residues and unlike Manuka honey, is not reliant on a particular botanical source [21]. SHRO elicits antimicrobial action due to the production of reactive oxygen species (ROS). ROS are produced from the oxidation of glucose in the presence of water, a reaction mediated by glucose oxidase (GOx) [23]. GOx is an enzyme synthesised naturally by bees and secreted into nectar and honey [24–26]. The biochemical engineering process enables the natural antimicrobial effects of honey to be tailored to different potencies and to provide a controlled, targeted delivery of ROS.

Currently, SHRO is available as a gel which uses the free water in wound exudate to catalyse ROS production. SHRO, like other ROS producing honeys, is very sticky and highly viscous, making clinical application to an infected wound difficult. Furthermore, SHRO is activated by the presence of free water which impacts long term stability. Modification of current formulations with a non-aqueous approach could result in an *in-situ* activated product with sustained ROS release, reduced application frequency and increased ease of handling [27]. One approach may be to formulate a non-aqueous product based on a non-aqueous system of emulsified SHRO droplets [28]. Emulsification in oil helps to protect the agent from premature ROS activation and provide a treatment option for minor topical infections such as that from a cut, scrape or burn.

Emulsions are known to be thermodynamically unstable systems with a propensity to quickly phase separate after manufacture in order to reduce

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the unfavourably high energy states associated with interfacial tension (IFT) [29]. Accordingly, previous SHRO emulsions have been stable for no more than 7 days [28]; however, a commercially viable emulsion-based product must be stable for much longer. The first aim of this study is therefore to quantify and evaluate the effect of processing conditions on emulsion stability and identify the set of parameters that produces the most stable emulsion. Stability of an emulsified product is largely driven by the energy input of the mixer, in this case the equivalent controlled variable being the rotational speed of the homogeniser rotor. This governs the shear stress exerted to create and disperse droplets of one phase within another [30]. According to Silva et al. [31] the more energy that a system is subjected to, the smaller the droplets formed. Stokes law (Eq. (1)) [32] states that a smaller particle diameter (r) induces a lower creaming/settling velocity (v). Settling velocity is calculated taking into account density differences ($\Delta\rho$) under the effect of gravity (g) and the viscosity of the continuous phase (η) [32,33]. Intuitively, the most stable emulsion might be formed by homogenisation at the highest rpm to form the smallest droplets [34], however the problem becomes complex when compromising for the temperature of the product, which must not exceed 55 °C. Above this temperature, GOx would denature, rendering the product ineffective.

$$v = \frac{2r^2(\rho_2 - \rho_1)g}{9\eta} \tag{1}$$

In addition to process optimisation to produce a stable, antimicrobial emulsion, this study aims to tackle the key issue of clinical applicability of SHRO by reformulating the product into a topical cream. Creams are non-Newtonian systems with a yield stress and viscoelastic response to shear during topical application, controlled by a complex microstructure [35]. An SHRO cream should provide the reduction in viscosity and cohesiveness to overcome the issues currently hampering clinical deployment of SHRO. Furthermore, a topical SHRO creams should impart a positive sensory perception in line with consumer expectations for a cream [36] for both clinician and patient, which is achieved through specific rheological characteristics. Therefore, the focus of this study is to formulate a cream system that enables easy delivery of ROS at concentrations capable of inducing a therapeutic antimicrobial response (>25 μM).

The elastic shear-response of a material is quasi-characterised by its storage modulus (G′), which corresponds to the system's storage of elastic energy [37]. The viscous shear-response is governed by the loss modulus (G″) that quantifies energy dissipation from unrecoverable viscous loss [38]. The energy stored and lost during a deformation cycle can be measured by calculating the loss tangent (tanδ), which is the ratio of G″ to G′ (Eq. (2)) [39]. At low shear rates a low phase angle (δ) is typically associated with a topical cream. Creams generally exhibit pseudoplastic behaviour (shear thinning), whereby viscosity monotonically decreases as a function of shear rate. As the application of shear stops, the viscosity of a cream recovers a transient manner to its initial state, known as thixotropy [40]. A low shear viscosity would allow for the product to maintain a high viscosity, whilst at higher shear rates viscosity is reduced, facilitating spreading action during application and/or evacuation from a container such as a squeezable tube [20].

$$\tan\delta = \frac{G'}{G''} \tag{2}$$

Active-containing creams must be formulated to enable a therapeutic response to the target area. As such emulsions with therapeutics residing in the dispersed phase must have an inherent mechanism for release and activation of the compound such as phase inversion which may be triggered by the introduction of shear flows, changing temperature, change in pH, the addition of salts or alterations in phase volumes in a process known as catastrophic phase inversion (CPI) [33]. This work is the first report of an engineered cream system to controllably deliver ROS to a wound site and demonstrate its ability of eradicating clinically relevant bacteria *in vitro* (Fig. 1).

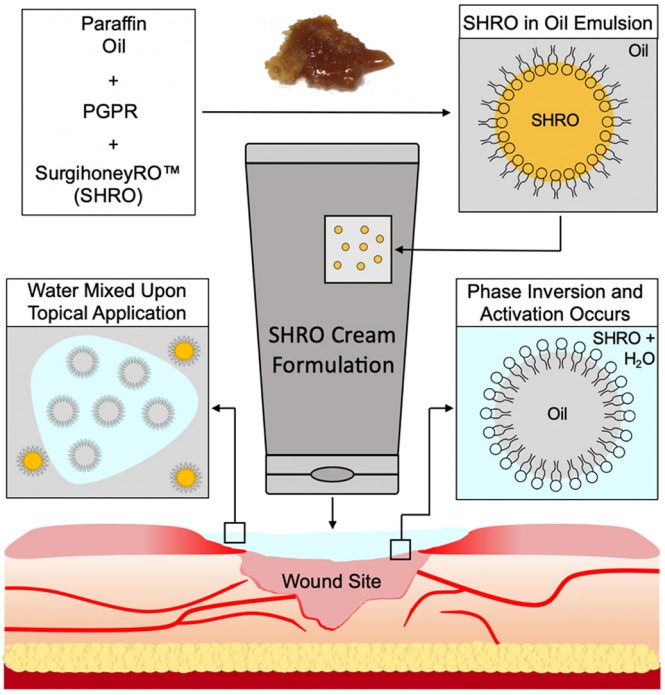

Fig. 1. Schematic diagram of the SHRO cream delivery system depicting the formulation, application and activation of the product.

2. Materials and methods

2.1. Materials

Analytical grade paraffin oil, XG and magnesium sulphate heptahydrate, Luria-Bertani (LB) broth and LB agar were supplied by Sigma Aldrich, UK. Polyglycerol polyricinoleate (PGPR) was supplied by Palsgaard, Denmark and SHRO was provided by Matoke Holdings, UK. Aerosil fumed silica (AR816) was supplied by Aerosil, UK and porcine skin was provided by Skin for Sale, UK. All materials were used as delivered with no further purification or modifications made.

2.2. Manufacture of emulsions

Firstly, a process optimisation study involving environmental temperature, shear rate and mixing time was caried out for the manufacture of 60% (v/v) SHRO emulsions described by Hall et al. was used to create emulsions. The first emulsions were manufactured as follows: PGPR was added to paraffin oil and homogenised at 10,000 rpm for 1 min using a T18 Ultra Turrax® mixer (IKA, UK). Maintaining a shear rate of 10, 000 rpm, SHRO was slowly added to the paraffin oil and PGPR dispersion using a 5 mL syringe. After complete addition of SHRO, homogenisation continued for 10 min. The temperature of the mixture was continuously monitored throughout the process using a digital thermocouple to monitor the temperature rise due to the energy input from the homogeniser. Emulsions were manufactured at room temperature and subsequently on ice to understand the effect of environmental temperature on the maximum temperature reached during processing. Subsequently, emulsions were manufactured on ice whilst varying the shear rate between 6000, 8000 and 10,000 rpm during and after addition of SHRO and mixing times for each shear rate were varied between 6, 8 and 10 min in order to understand the effect of these processing parameters on maximum temperature recorded during manufacture, and the stability of the final emulsion.

2.3. Visual characterisation of emulsion stability

Emulsions were examined over a 7-day period at 21 °C with images taken at 24, 48, 72 and 168 h using an iPhone X camera from a distance

of 15 cm and at 1.0 x zoom. Image J software (1.47v National Institutes of Health, USA) was used to obtain a measurement of any separation that had occurred, quantified by the presence of a fixed scale placed next to the sample during imaging. Samples were measured in triplicate with mean and standard deviation of the height of the separated phase reported. Emulsions were also visually assessed to determine if any phase separation processes occurred.

2.4. Cream formulation

Creams were formulated based on 60% (*v*/v) SHRO emulsions formed following the optimum protocol found as a result of the process optimisation outlined in Section 2.2, with the addition of either fumed silica (AR816 Aerosil) or xanthan gum (XG) as a thickener. Creams containing fumed silica were manufactured by first dispersing the silica in paraffin oil under high shear (10,000 rpm) at concentrations of 1 (SIL1) and 2% (*v*/v, SIL2), after dispersion of PGPR. Manufacture of a third cream formulation (XG1) containing XG as the thickener began with dissolving 0.5% (*w/v*) XG in glycerol using the homogeniser at 10,000 rpm. Subsequently, the XG-glycerol solution was allowed to cool. SHRO was then added to the XG-glycerol solution, forming what would become the dispersed (W in W/O) phase of the cream, containing SHRO and XG-glycerol solution in a 1:1 ratio. Following the emulsion manufacture protocol outlined in 2.2, the SHRO, XG and glycerol mixture was added using a 5 mL syringe in place of pure SHRO, meaning that this cream had half the quantity of active SHRO compared to the emulsions and silica creams. A fourth cream (XG2) was manufactured following the methodology for XG1, with the addition and dissolution of 0.5% (*w/v*) magnesium sulphate heptahydrate simultaneously with the dissolution of XG in glycerol.

2.5. Droplet size analysis of dispersed phase

Laser diffraction measurements were recorded by a Malvern 3000 Mastersizer (Malvern Instruments, UK). Refractive indexes (RI) of 1.487 and 1.473 were used for SHRO and paraffin oil, respectively. RI was determined using an ORA-3HA refractometer (Kern-Sohn, Germany) and manufacturer provided values. The software used to calculate size distribution assumed spherical droplets in a uniform media. Size distribution was measured on days 0 and 7 to investigate the occurrence of coalescence. Each emulsion ratio was measured in triplicate and with three readings per experimental run.

2.6. Rheological characterisation of SHRO creams

To determine the viscoelastic properties of formulated SHRO creams, an AR-G2 rheometer (TA Instruments, UK) with sandblasted parallel plates (diameter = 40 mm, gap height = 1 mm) was used. Flow curves were determined by measuring the viscosity over a rotational flow ramp from shear rates of 1.0 to 200.0 s^{-1} over a 5-min period. An oscillatory strain sweep with the frequency set at 1 Hz was then utilised to determine the linear viscoelastic region (LVR) of all cream samples, over which frequency sweeps between 0.1 and 100 Hz were conducted at a strain of 0.5% to obtain viscoelastic behaviour profiles of storage (G′) and loss (G″) moduli. Furthermore, thixotropy was evaluated by using a 3 interval thixotropy test to measure viscosity after a step change in shear rate from 1.0 s^{-1} for 60 s to 100 s^{-1} for 30 s.

2.7. Hydrogen peroxide release from SHRO emulsions and creams

As an indicator of formulation efficacy, a fluorescent assay kit (Sigma Aldrich, UK) was used to quantify production of hydrogen peroxide (H_2O_2). A calibration curve was first produced using 10, 3, 1, 0.3, 0.1, 0.03, 0.01 and 0 μM H_2O_2 standards. 1 mL of each cream sample was added to 2 mL of deionised water and vortexed for 10 s. 0.1 mL of diluted cream was then added to a further 30 mL of deionised water (1:300) in order to conduct the assay. This was repeated at each time point (24, 48, 72 and 168 h). Manufacturer's assay protocols were followed throughout, briefly standard solutions were made which included: 50 μL of red peroxidase substrate master mix, 200 μL of 20 units/mL peroxidase and 4.75 mL of assay buffer. 50 μL of sample or standard was pipetted into each well with 50 μL of master mix subsequently added. Wells were then mixed, protected from light and incubated for 30 min at room temperature. A Tecan Spark plate reader (Tecan Trading AG, Switzerland) was used to measure fluorescence intensity (λ_{ex} = 540 nm, λ_{em} = 590 nm).

2.8. In vitro antimicrobial efficacy of SHRO creams

LB broth and LB agar were diluted to concentrations of 20 g/L and 35 g/L respectively and sterilised in an autoclave at 121 °C and 100 kPa for 20 min.

Overnight cultures of inoculated broth containing *Staphylococcus aureus* ATCC 29213 were used. The optical density of the culture was measured using a Spectronic Helios Gamma UV–Vis Spectrophotometer at 600 nm (Thermo Fisher Scientific, UK). Prior to inoculation, the culture was then diluted to and optical density of 0.04, following spread onto a sterile LB agar plate a well was created in the centre of the inoculated agar using a 10 mm hole borer. Cream samples were then prepared by diluting 1 mL of each cream with 2 mL of deionised water, vortexed for 1 min to trigger ROS production and 250 μL added into the agar well. and incubated for 24 h at 37 °C. Zones of inhibited bacterial growth were measured after cultivation for 24 h at 37 °C with the well diameter deducted from the zone size.

2.9. Texture analysis

Texture analysis was carried out to study the cohesiveness of SHRO creams compared to the commercially available product. A TA.XTPlus Texture Analyser (TA Instruments) was employed, with a 30,000 g capacity load cell and 6 mm diameter cylindrical stainless steel probe. 2 g of each cream was placed into the sampling area and the probe lowered into to a distance of 0.1 mm from the base. Any excess sample was removed using a plastic spatula. The load cell was then lifted at a constant rate and the resistive force exerted on the probe, attributed to the cohesion within the honey-containing formulations, was measured until point of failure using the Stable Micro Systems Exponent software (TEE32 version 6).

2.10. Contact angle

Single drops of cream were dropped using a syringe onto porcine skin at both a horizontal orientation and at an angle of 45° with respect to the horizontal axis. Droplets were imaged using an iPhone X camera at 1.0 x zoom and analysed for static contact angle using Image J software (1.47v National Institutes of Health, USA).

2.11. Statistical analysis

All emulsions and creams were manufactured in triplicate as were all measurements taken. GraphPad Prism® 5.0 software was used to perform statistical analysis. *t*-Tests were used to determine significance at a 0.05 level. *p* values are indicated as follows $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

3. Results

3.1. Process optimisation for 60% SHRO emulsions

To optimise the processing of 60% SHRO emulsions, an initial process analysis was performed (Fig. 2). When homogenised at 10,000 rpm for 10 min at room temperature, it was found that the temperature during processing reached a maximum of 78.8 ± 2.1 °C, exceeding 55 °C, the denaturation temperature of the enzyme glucose oxidase (GOx). In comparison when the same emulsion was formulated within an ice bath the maximum

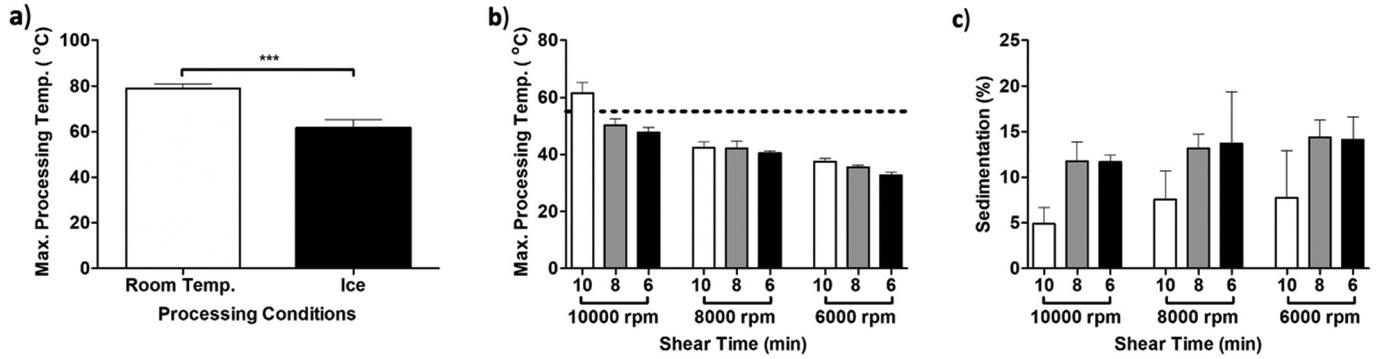

Fig. 2. Investigation into the processing conditions for a 60% SHRO emulsion: (a) influence of manufacturing emulsions on ice compared to room temperature on the maximum temperature observed during processing; (b) effect of mixing time and shear rate on maximum processing temperature measured when formulating emulsions on ice, the dashed line in panel b indicates the denaturation temperature of GOx; (c) and the effect of shear time and rate on emulsion stability after 7 days when emulsions are formulated in an ice bath.

temperature reached during mixing was significantly ($p < 0.0001$) reduced to 61.5 ± 3.7 °C (Fig. 2a).

The effects of different mixing times and shear rates on the maximum processing temperature recorded during manufacture of emulsions on ice are shown in Fig. 2b, with the denaturation temperature of GOx marked as the dashed line. It was found that a reduction in shear rate from 10,000 rpm resulted in lower maximum recorded temperatures of the emulsions, remaining below the denaturation temperature of GOx, with a maximum of 42.3 ± 2.1 °C measured at 8000 rpm. Additionally, a reduction in mixing time from 10 to 8 min whilst applying a shear rate of 10,000 rpm resulted in the maximum recorded temperature remaining below that of the denaturation temperature of GOx, at 50.3 ± 2.2 °C. Overall, lower shear rates resulted in lower maximum temperatures of the emulsions, as did a reduction in mixing time, except for mixing at 8000 rpm for 10 and 8 min which were not significantly different ($p = 0.92$).

However, Fig. 2c highlights that a decrease in shear rate and shear time appears to come at the cost of stability of the final product, with greater phase separation occurring for the emulsions processed at lower shear rates and mixing times. Overall, an emulsion produced on ice from homogenisation at 8000 rpm for 10 min was found to be optimum in terms of achieving maximum stability whilst not exceeding the denaturation temperature of GOx.

3.2. Cream formulation stability

Creams were manufactured in line with the optimum processing conditions found in Section 3.1 (homogenisation at 8000 rpm for 10 min). Droplet size (D_{10}, D_{50}, and D_{90}) and distribution (DSD) was analysed for the dispersed phase of each different cream formulation immediately after manufacturing. Fumed silica creams exhibited smaller droplet sizes and a narrower (DSD) compared to formulations thickened using XG. SIL1 and SIL2 creams were found to have average median diameters (D_{50}) of 2.7 and 2.2 μm respectively (Table 1). In contrast XG-based creams produced a broader DSD with much greater median diameters of 79.4 μm (XG1) and 47.4 μm (XG2) (Table 1). Since droplet size is directly related to settling velocity according to Stokes law and therefore cream stability, fumed silica creams were deemed superior to XG-thickened creams in terms of stability.

Table 1
Droplet sizes of Aerosil fumed silica creams (SIL1 and SIL2), and xanthan gum creams (XG1 and XG2).

Cream formulation	Size (μm)		
	D_{10}	D_{50}	D_{90}
SIL1	1.4	2.7	5.0
SIL2	0.7	2.2	7.1
XG1	35.5	79.4	141.3
XG2	25.1	47.4	112.2

3.3. Cream antimicrobial efficacy

In addition to having stability as a final product, it is imperative that cream formulations maintain the antimicrobial efficacy of SHRO emulsions previously demonstrated by Hall et al. [28]. Therefore, the levels of hydrogen peroxide production following phase inversion were evaluated, and results presented in Fig. 3a. Fumed silica creams SIL1 and SIL2 produced hydrogen peroxide concentrations of 1.4 ± 0.5 μmol g^{-1} and 1.2 ± 0.3 μmol g^{-1} respectively, over a 24-hour period. This was similar to those formulated using xanthan gum, XG1 and XG2 at 1.5 ± 0.4 and– 1.4 ± 0.07 μmol g^{-1} respectively. However, over a 7-day period, Aerosil formulations clearly outperformed XG formulations, with SIL1 and SIL2 found to produce 12.1 ± 0.5 and 11.5 ± 0.4 μmol g^{-1} of hydrogen peroxide respectively, in clear contrast to 3.1 ± 0.2 μmol g^{-1} by XG1 and 2.4 ± 0.4 μmol g^{-1} by XG2 (Fig. 3a). Despite these differences in released H_2O_2 concentrations, all creams were found to inhibit the growth of *S. aureus*. That said, both fumed silica creams (SIL1 and SIL2) produced a zone of inhibition 42% larger than creams containing xanthan gum (XG1 and XG2), as shown by Fig. 3b.

3.4. Rheological and textural characterisation of SHRO creams

Since SIL1 and SIL2 were had lowest droplet sizes, DSDs and were the most efficacious in antimicrobial performance, these formulations were selected for further rheological characterisation (Fig. 4). Both cream formulations exhibited pseudoplastic flow with a steady decrease in viscosity as shear rate increased. Increasing silica content increased apparent viscosity at all measured shear rates, for example the low shear apparent viscosity of SIL2 was 20.4 ± 1.4 Pa s at a shear rate of 10/s, compared to 12.1 ± 1.1 Pa· s for SIL2 (Fig. 4a). Analysis of the viscoelastic properties of the creams (Fig. 4b) determined that SIL1 displayed higher storage (G′) than loss (G″) moduli values across all tested frequencies (0–100 Hz). In comparison, G′ was only found to be greater than G″ in SIL2 at frequencies of 25 Hz (1060.3 ± 1.2 Pa and 1046.9 ± 2.6 Pa respectively) and below, Above 25 Hz, G″ becomes dominant (Fig. 4b). Further characterisation by texture analysis found that cream formulations demonstrated significantly lower degrees of cohesiveness than the commercial SHRO product in comparison to the commercial SHRO product (79.87 N) with SIL1 and SIL2 producing maximum cohesive forces of 15.26 N and 22.42 N respectively.

3.5. Application performance studies

Thixotropy and contact angle are important factors for the application of a topical cream. When subjected to a step change from a low (1.0 1/s)

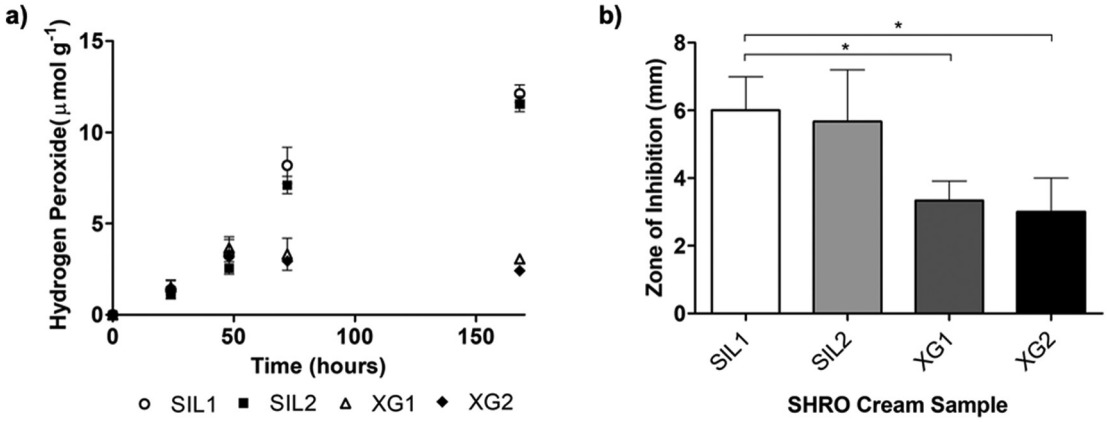

Fig. 3. Normalised hydrogen peroxide production (a) and zones of inhibition for *S. aureus*, treated by inverted Aerosil fumed silica (SIL1, SIL2) and xanthan gum creams (XG1 and XG2) (b).

to a higher shear rate (100 1/s), apparent viscosity reduced significantly following the pseudoplastic behaviour observed in Fig. 4a, but typically recovers within a few seconds. Therefore, both silica creams exhibit a high degree of thixotropy. Unlike SIL1, SIL2 exhibited hysteresis upon the application and removal of shear as indicated by the recovered viscosity being lower than the initial low shear measurements. After 3 cycles, the viscosity of SIL2 was found to be 20% less than its initial (0–60 s) viscosity (Fig. 5a). Static contact angles for both creams were found to be <90° indicating favourable wetting of the surface and also demonstrated higher contact angles on porcine skin orientated at 45° with SIL1 and SIL2 showcasing contact angles of 45.2 ± 3.4° and 58.6 ± 2.6° respectively. Overall SIL1 displayed significantly lower contact angles than that of SIL2 in both scenarios, with contact angles measured at 33.8 ± 3.3° and 45.0 ± 2.8° respectively on flat porcine skin (Fig. 5b).

4. Discussion

This work focused on the development of the 60% SHRO emulsion system first manufactured by Hall et al. [28] into an antimicrobial topical cream. This emulsion was chosen as it exhibited greater stability compared to other emulsions with differing ratios of SHRO to paraffin oil and contained the most active ingredient out of all emulsions previously studied.

In order to further develop and improve the 60% SHRO emulsion system proposed by Hall et al. [28], the emulsification process was scrutinised first. When preparing the emulsions, an UltraTurrax rotor-stator homogeniser was used to form small droplets of the aqueous SHRO phase and effectively disperse them throughout the continuous paraffin oil phase. This homogeniser is commonly used in literature to create liquid dispersions, as summarised by Kempin et al. [41]. The rotor-stator homogeniser imparts a high amount of mechanical energy that is dissipated as heat and can therefore lead to an excessive temperature increase and associated destabilisation of proteins [42]. In this case, that would be the denaturation of the enzyme GOx at temperatures greater than 55 °C [28,43]. This enzyme is responsible for the oxidation of glucose and the subsequent production of hydrogen peroxide, which is necessary for the product to elicit an antimicrobial effect [44]. In theory, processing above 55 °C could therefore hinder efficacy and affect the ability to topically administer a uniform dose [43]. It was demonstrated that the maximum temperature recorded when manufacturing SHRO emulsions under ambient conditions exceeded 55 °C (Fig. 2a). During initial optimisation of the emulsion formulation by Hall et al. [28] the antimicrobial efficacy of the emulsions were tested against an equivalent dilution of SHRO where it was demonstrated that in most cases there was no apparent reduction in inhibitory ability. This technique however is low in resolution and, at lower concentrations (30% SHRO) against gram negative bacteria, the equivalent dilution was found to inhibit growth, but the emulsion did not. This may, at least in part, be due to the processing conditions. Therefore, inspired by the work of Peng et al. [45], an ice bath was used during homogenisation for all subsequent emulsions, resulting in a 22% reduction in maximum processing temperature, which remained below 55 °C (Fig. 2a).

Further investigation into the processing conditions during emulsification of SHRO in paraffin oil concerned the effect of shear rate during homogenisation and the mixing time on processing temperature and emulsion stability (Fig. 2b and c). As expected, a reduction in both shear rate and mixing time were found to reduce the maximum recorded temperature during homogenisation as each factor change results in a lower total energy input to the system. However, a compromise had to be found with stability, since a reduction in shear rate and homogenisation also increased the degree of sedimentation, with an 55.2% increase when shear was reduced from 10,000 rpm (4.88 ± 1.8%) to 8000 rpm (7.57 ± 3.1%) and a 140.9% increase when the mixing time was reduced from 10 min (4.88

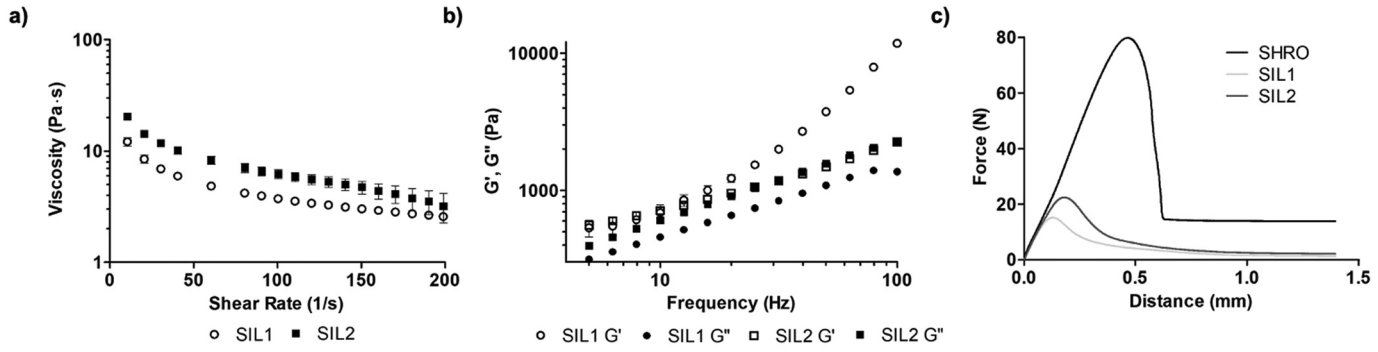

Fig. 4. Flow curves (a) and frequency sweeps (b) to assess the rheology of SHRO creams and cohesive force-distance curves (c) of SHRO creams and the commercially available SHRO product obtained using a texture analyser.

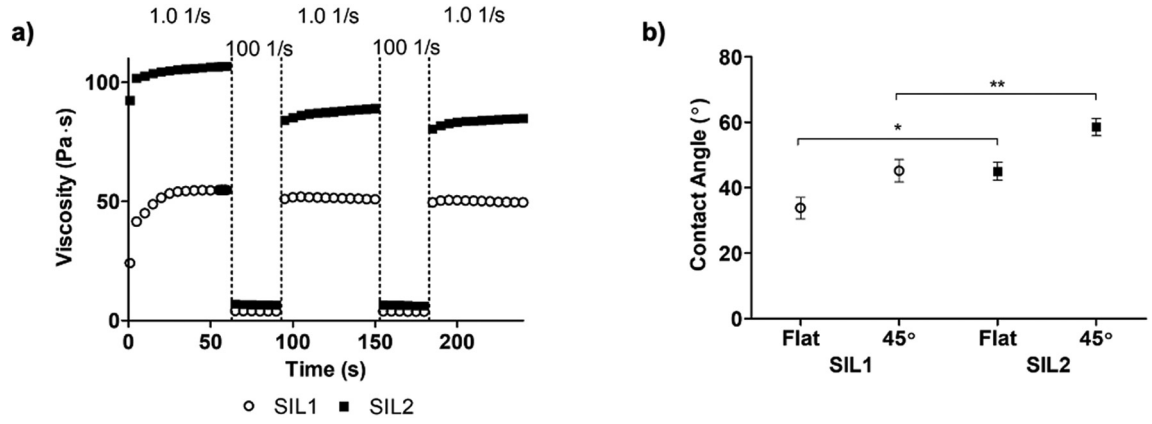

Fig. 5. Thixotropic flow curves of SIL1 and SIL2 creams over a 3-interval step change from low (1.0/s) to high (100.0/s) shear (a) and static contact angles of cream droplets on porcine skin at 0° (flat) and 45° orientations with respect to the horizontal axis (b).

± 1.8%) to 8 min (11.75 ± 2.1%) (Fig. 2c). This was in line with expectations since a lower mechanical energy input, controlled by the rotor rpm, results in a lower shear stress exerted on droplets and a larger droplet size, which according to Stokes law results in a higher settling velocity and therefore faster phase separation [32]. Therefore, optimum homogenisation conditions were determined to be 8000 rpm for 10 min, in terms of achieving maximum stability whilst not exceeding the denaturation temperature of GOx. Considering the occurrence of sedimentation in various SHRO emulsions, it is recommended that this risk of phase separation is mitigated in future SHRO emulsion-based products by imposing a shake-before-use directive, providing droplets can easily be re-dispersed without causing coalescence. For a viscous topical cream, this is unlikely to be effective; however the risk of phase separation is to be further mitigated through addition of a thickener.

Product development continued with 60% SHRO emulsions manufactured on ice with a homogenisation step of 8000 rpm for 10 min. Cream formulations incorporated 1% (SIL1) and 2% (SIL2) Aerosil fumed silica to thicken the paraffin oil-based continuous phase of the emulsion or XG (XG1) to thicken the active-containing dispersed phase of the emulsion to achieve heightened stability and rheological properties reminiscent of a typical cream [16]. Magnesium sulphate heptahydrate ($MGSO_4{\cdot}7H_2O$) was added to one formulation of xanthan gum cream (XG2) with the aim of reducing polymer entanglements between the glycerol and thickening agent, in line with methodology proposed by Ciullo, et al. [46].

Firstly, the size of SHRO droplets dispersed throughout the continuous phase was investigated, which as discussed previously, correlates to stability [28]. Droplet sizes measured in the fumed silica creams were similar to those of the original SHRO emulsions at approximately 2.5 μm in median diameter [28], whilst XG-based creams produced wider particle size distributions with median diameters of 79.4 μm (XG1) and 47.4 μm (XG2) (Table 1). One of the issues highlighted in the development of the SHRO emulsion was that of sedimentation. Although Stokes' law dictates that with a larger droplet size comes greater instability, the incorporation of a thickener into a cream increases the bulk viscosity when compared with SHRO emulsions, and creams therefore exhibited a slower rate of sedimentation [32,33]. This was reflected in that no sedimentation was observed in any cream formulation after 1 month of storage at 37 °C. Therefore, incorporating fumed silica or XG into the formulations significantly increased emulsion stability.

The development of an SHRO cream came with the intention to trigger the activation of ROS *in situ* through catastrophic phase inversion. In order to assess that this phenomenon still occurred when the creams were subjected to water and shear, a hydrogen peroxide assay was conducted. It was found that all creams; SIL1 (1.4 ± 0.5 μmol g^{-1}), SIL2 (1.2 ± 0.3 μmol g^{-1}), XG1 (1.5 ± 0.4 μmol g^{-1}) and XG2 (1.4 ± 0.01 μmol g^{-1}) produced similar amounts of hydrogen peroxide over a 24-hour period (Fig. 3a). However, concentrations of hydrogen peroxide produced by creams required 48 h to reach a comparable level to that produced by the equivalent 60% SHRO emulsion after 24 h (2.2–4.2 μmol g^{-1} and 4.2 ± 0.2 μmol g^{-1} respectively). This implies that the addition of a thickening agent reduces the rate at which hydrogen peroxide is produced. It was also demonstrated that whilst both fumed silica creams, SIL1 (12.1 ± 0.5 μmol g^{-1}) and SIL2 (11.5 ± 0.4 μmol g^{-1}), produced similar amounts of hydrogen peroxide over a 7-day period. Those formulated using XG, XG1 (3.1 ± 0.2 μmol g^{-1}) and XG2 (2.4 ± 0.4 μmol g^{-1}), produced significantly less H_2O_2. This is likely due to the addition of glycerol into the dispersed phase, which is required in these formulations to dissolve the XG, hence there is a lower quantity of SHRO within these creams. However, despite producing differing amounts of hydrogen peroxide, all creams were found to inhibit the growth of *S. aureus*, a clinically-relevant bacterial strain [2,21,27]. Fumed silica creams, however, exhibited significantly larger zones of inhibition (42%) than XG formulations (Fig. 3b).

Due to the significantly greater potency exhibited by the fumed silica containing creams, further work focused on the characterisation of these formulations. Rheological measurements indicated that both SIL1 and SIL2 exhibited pseudoplastic flow, demonstrating a reduction in viscosity with an increase in shear, a characteristic ideal for cream application (Fig. 4a). At low shear (1.0/s), creams with a higher concentration of fumed silica displayed higher viscosities as expected, with SIL2 exhibiting a viscosity of 20.4 ± 1.4 Pa·s compared to 12.1 ± 1.1 Pa·s for SIL1. Low shear viscosity is important in determining the mode of dispensing the product; a cream with a higher low shear viscosity may be more suitable to storage in a pot. A greater rate of decrease in viscosity was observed at lower shear rates, which are known to deconstruct random polymer entanglements. At higher shear rates of approximately 150/s, a slight increase in rate of viscosity reduction was observed which is likely to be attributable to the cleavage of hydrogen bonds and the breakage of Van der Waals interactions [47]. Oscillatory frequency sweeps further elucidated the viscoelastic properties of the cream. G′ and G″ were found to increase with frequency; an effect attributed to the dissipation of energy within the droplets (Fig. 4b) [48,49]. SIL1 displayed higher G′ than G″ values across all tested frequencies. This indicated that under all tested conditions the formulations retained a viscoelastic solid structure. However, although SIL2 creams demonstrated comparable G′ values to SIL1 at low frequencies (25 Hz), at 40 Hz both G′ (1060.3 ± 1.2 Pa) and G″ (1046.9 ± 2.6 Pa) displayed similar values, which indicates a breakdown of the microstructure (Fig. 4b) [50]. This is not ideal since it may increase variability of performance if this formulation is applied by a patient or clinical practitioner at different rates. Furthermore, data from the texture analyser indicated that although cream formulations have a significantly lower cohesiveness than the SHRO™ sachet product (79.87 N), SIL2 produced a higher degree of cohesiveness (22.42 N) than the SIL1 formulation (15.26 N) (Fig. 4c). Reduced cohesiveness of the creams will allow for delivery of SHRO in a less sticky form compared to SHRO which is comparable in cohesiveness to

commercial honey. This is anticipated to ease clinical application, increasing patient satisfaction.

Further studies focused on application in order to establish how the creams would be retained on a topical surface. Additional rheological studies scrutinising the thixotropy of the creams Furthermore, when creams were rapidly switched from a low (1.0/s) to a higher shear rate (100/s), the viscosity reduced significantly but quickly recovered when reverted back to low shear. This is a characteristic that would promote ease of delivery to the application site where viscosity recovery would facilitate product retention locally. However, unlike SIL1, SIL2 did display hysteresis upon the application and removal of shear to a greater extent than the SHRO emulsion (Fig. 5a). This could imply a permanent breakdown of microstructure, and therefore a transient reduction in ability to be retained on the skin [51]. The high shear rate of 100/s in this experiment is considerably lower than those estimated to occur during make-up application [52], however it was assumed that cream might be applied to a sensitive wound more carefully, inducing lower shear rates.

Contact angle measurements were also taken in order to elucidate wettability and spreadability on a topical surface. Cream formulations demonstrated a higher contact angle when porcine skin was oriented at 45° compared to the flat surface due to gravitational influences. Contact angles were low (< 90°) for both formulations as expected, given the continuous phase of paraffin oil and the lipophilic surface of the skin (Fig. 5b). This signifies that wetting of the pig skin surface is favourable. The striking similarities between porcine skin and human skin enable extrapolation of results to indicate that the cream formulation would exhibit good spreadability on human skin [53]. This is vital for sensory purposes of a topical product and also for efficacy, increasing surface area for delivery of ROS [54]. SIL1 demonstrated significantly lower contact angle than SIL2 which indicates that increasing Aerosil content decreases contact angle on pig skin and therefore spreadability. This is expected to be due to the associated increment in viscosity of the cream droplet, which is known to increase contact angle [55]. This suggests that SIL1 would be the superior formulation in terms of spreadability and therefore ease of clinical application and patient satisfaction.

Overall, all cream formulations demonstrated significantly higher stability than SHRO emulsions. Aerosil was the preferred thickening agent due to the heightened antimicrobial efficacy *in vitro* compared with XG-thickened creams. Both 1% and 2% Aerosil formulations demonstrated rheological behaviour that is beneficial of a topical cream. Application studies revealed that 1% Aerosil, SIL1, was the optimum formulation due to reduced cohesiveness and lower contact angle, which translate to improved ease of application, spreadability and ultimately, efficacy.

5. Conclusions

This research aimed to address the need for alternative topical infection treatments to current antibiotics by reformulating SHRO into a topical cream that produced reactive oxygen species *in situ*. This was a significant development acting to improve clinical applicability of SHRO, currently hampered by its stickiness, high viscosity and stability (arising from premature activation by free water). Cream formulations containing both fumed silica and xanthan gum were shown to retain antimicrobial efficacy *in vitro* against clinically relevant *S. aureus*. However, formulations functionalised with fumed silica demonstrated an ability to generate more ROS (12.1 ± 0.5 μmol g^{-1}) than those containing xanthan gum (3.1 ± 0.2 μmol g^{-1}) and displayed greater efficacy *in vitro*.

Material properties of fumed silica formulations (both 1 and 2%) were assessed using rheological and texture analysis techniques to determine the optimum topical cream. It was shown that formulations exhibited pseudoplastic flow, demonstrating a reduction in viscosity with an increase in shear, a characteristic ideal for cream application. However, at higher frequencies (40 Hz) creams formulated with 2% fumed silica indicated a breakdown in microstructure which could introduce variability in performance. Texture analysis determined that 1% fumed silica formulations produced lower cohesiveness and would allow for delivery of SHRO in a less sticky form, easing clinical application, increasing patient satisfaction and reduce product waste. This conclusion was supported by contact angle measurements which indicated good topical spreadability and favourable wetting of the surface.

Credit authorship contribution statement

Connor O'Farrell*: Conceptualisation, data curation, formal analysis, investigation, methodology, project administration, writing – original draft preparation, writing – review and editing.

Thomas J. Hall: Conceptualisation, data curation, formal analysis, investigation, methodology, project administration, writing – original draft preparation, writing – review and editing.

Liam M. Grover: Supervision, funding acquisition.

Sophie C. Cox: Conceptualisation, project administration, supervision, writing – review and editing.

All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors have no conflict of interests to declare.

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