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DOI:

[10.1016/j.msec.2019.05.020](https://doi.org/10.1016/j.msec.2019.05.020)

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Document Version

Peer reviewed version

Citation for published version (Harvard):

Hall, T, Blair, J, Moakes, R, Pelan, E, Grover, L & Cox, S 2019, 'Antimicrobial emulsions: formulation of a triggered release reactive oxygen delivery system', *Materials Science and Engineering C*, vol. 103, 109735. <https://doi.org/10.1016/j.msec.2019.05.020>

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Checked for eligibility: 17/05/2019

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Accepted Manuscript

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PII: S0928-4931(18)33131-X
DOI: <https://doi.org/10.1016/j.msec.2019.05.020>
Reference: MSC 9735

To appear in: *Materials Science & Engineering C*

Received date: 12 October 2018

Revised date: 15 March 2019

Accepted date: 8 May 2019

Please cite this article as: T.J. Hall, J.M.A. Blair, R.J.A. Moakes, et al., Antimicrobial emulsions: Formulation of a triggered release reactive oxygen delivery system, *Materials Science & Engineering C*, <https://doi.org/10.1016/j.msec.2019.05.020>

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Antimicrobial Emulsions: Formulation of a Triggered Release Reactive Oxygen Delivery System

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Abbreviations: SurgihoneyRO (SHRO), reactive oxygen species (ROS), antimicrobial resistance (AMR), methicillin-resistant staphylococcus aureus (MRSA), oil-in-water (O/W), water-in-oil (W/O), critical phase inversion (CPI), polyglycerol polyricinoleate (PGPR), linear viscoelastic region (LVR), Luria-Bertani (LB), Staphylococcus aureus (S. aureus), Escherichia coli (E. coli), Pseudomonas aeruginosa (P. aeruginosa).

Abstract

The enzyme glucose oxidase mediates the oxidation of glucose to produce reactive oxygen species (ROS), such as hydrogen peroxide. This reaction and its products are key to providing honey with its antimicrobial properties. Currently, honey is an adherent, highly viscous product that produces ROS by means of a water-initiated reaction. These properties reduce clinical usability and present a formulation problem for long term stability. This study aims to engineer a water-in-oil emulsion containing an engineered honey (SurgihoneyRO™) that is easy to administer topically and is controllably activated *in-situ*.

Paraffin oil continuous emulsions formulated using the emulsifier polyglycerol polyricinoleate displayed shear-thinning characteristics. Viscosities between 1.4 and 19.3 Pa.s were achieved at a shear rate representative of post-mixing conditions (4.1 s^{-1}) by changing the volume of the dispersed phase (30 – 60%). Notably, this wide viscosity range will be useful in tailoring future formulations for specific application mechanisms. When exposed to water and shear, these emulsion systems were found to undergo catastrophic phase inversion, evidenced by a change in conductivity from $0\text{ }\mu\text{S}$ in the non-aqueous state, to $>180\text{ }\mu\text{S}$ in the sheared, inverted state. Encouragingly, sheared formulations containing $\geq 50\%$ SurgihoneyRO™ generated sufficient levels of ROS to inhibit growth of clinically relevant Gram-positive and Gram-negative bacteria.

This study demonstrates an ability to formulate ROS producing emulsions for use as an alternative to current topical antibiotic-based treatments. Promisingly, the ability of this system to release water-sensitive actives in response to shear may be useful for controlled delivery of other therapeutic molecules.

Keywords: Surgihoney, SurgihoneyRO, Honey, Emulsion, Inversion, ROS, AMR, Antimicrobial, Formulation, CPI.

1. Introduction

Bacteria and other resistant pathogens are predicted to kill more people than cancer by 2050, with estimated global deaths in excess of 10 million per year [1,2]. Antimicrobials are key to infection prevention and curative treatments, for example protecting patients exposed to complex wound healing procedures, such as surgery or cancer chemotherapy [3,4]. Therefore, research into novel antimicrobials and systems by which to deliver them is a healthcare priority [5].

With the antibiotic development pipeline running dry, many researchers are continuing to look for inspiration from nature, such as the use of reactive oxygen species (ROS). This includes the use of hydrogen peroxide (H_2O_2) to treat infections. However, due to its reactivity high doses of H_2O_2 are often used ($\geq 3\%$) to keep the level of ROS above the bactericidal concentration [6]. This can have cytotoxic effects on any surrounding cells [7,8]. To combat this, Zhu *et al.* concluded that successful delivery of a lower, tailored dose of ROS would be advantageous [9].

The use of ROS to kill bacteria is a defence mechanism found in honey to prevent it from spoiling. Honey has been used for thousands of years for topical wound care applications without the development of resistance [10]. The problem with using natural honey as an antimicrobial is that the effect can differ greatly from batch to batch [11]. This is largely due to the conditions within the beehive, which are influenced by a variety of environmental factors such as; the weather, accessibility to pollen and humidity [12]. As such, to realise the potential use of honey as an alternative antimicrobial it would be necessary to chemically engineer a product that would deliver a controlled and tailorable amount of ROS over a sustained period.

There are a number of medical grade honeys available for use, including Medihoney manuka honey, Activon manuka honey and engineered SurgihoneyRO™ (SHRO). Promisingly, *in vitro* and *in vivo* studies using SHRO have demonstrated the complete eradication of the infecting bacterial culture [13–17]. This included both Gram positive and negative species as well as drug resistant strains, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* [17]. In addition, it has been shown to be effective against fungi and prevented or reduced the seeding of biofilms [14,15]. Furthermore, SHRO has been found to be equally or more efficacious than Medihoney manuka honey and Activon manuka honey as well as two of the most commonly used topical wound care alternatives, iodine and silver [13,16]. Interestingly, in contrast to iodine and silver dressings, SHRO was not shown to elicit cytotoxic effects [13].

SHRO is currently packaged as a raw product in a sachet or tube. This delivery system is clinically non-optimal since it is highly viscous and adherent to surfaces. Therefore, the development of alternative physical formulations, which incorporate SHRO and facilitate an application specific release profile of ROS, is of great interest. When designing such a system it is important to consider the underlying mechanisms of action. In the case of SHRO, the oxidation of glucose to produce ROS is a reaction that requires water (Figure 1a). SHRO contains 16-20% water but this is bound to sugars and is not free to react [18]. As a consequence, to avoid premature production of ROS before clinical application a non-aqueous vehicle is required. Such a formulation would enable the product to be stored and activated *in-situ*. To improve the ease of handling SHRO, and facilitate controlled release of ROS, this non-aqueous vehicle will require appropriate physical structuring. One such approach may be to create an emulsion that incorporates SHRO into its non-aqueous phase (Figure 1b). An

emulsion can be defined as a dispersion of droplets of one liquid in another, in which it is not soluble or miscible [19].

There are two basic forms of emulsions: (1) oil in water, denoted (O/W) and (2) water in oil (W/O). Emulsions are inherently thermodynamically unstable and systems quickly phase separate after manufacture to reduce the unfavourably high energy states associated with interfacial tension [20]. To improve stability over time, emulsifiers or surfactants have to be used, these lower the tension between the two species allowing the persistence of droplets, which may contain actives, over much longer time scales [21].

Phase inversion is a phenomenon that occurs when the dispersed phase of an emulsion becomes the continuous phase or vice versa, during this process surfactants spontaneously change their arrangement [22]. Phase inversion is widely used to formulate emulsions, with many manufacturers using this process in order to create products with increased stability and a small size distribution of droplets within a continuous phase [23]. It can occur due to: the introduction of particular flows (shear induced), changing temperature, alterations in phase volumes (known as catastrophic phase inversion (CPI)), change in pH or the addition of salts [24].

The focus of this study is to formulate an emulsion system that enables easy delivery of ROS producing honey as a novel antimicrobial (Figure 1). It is hypothesised that by incorporating the SHRO in oil, this will help to protect the agent from premature ROS activation. By optimising the dispersion of SHRO in paraffin oil, shear thinning behaviour could be achieved easing application. (Figure 1). This work aims to use an inversion trigger whereby water and shear are added to the system until the emulsion inversion point is exceeded and CPI occurs. This paper is the first report of an engineered emulsion system to controllably deliver ROS and demonstrates it is capable of eradicating clinically relevant bacteria *in-vitro*.

Figure 1. Schematic highlighting the production of hydrogen peroxide and reactive oxygen species through the aerobic glucose oxidase mediated oxidation reaction of glucose and water (a). The key components of a SurgihoneyRO™ in oil emulsion (b), in which SHRO is the dispersed phase (c). After addition of water and shear (d) phase inversion of the emulsion occurs (e) allowing for the production of hydrogen peroxide and other reactive oxygen species.

2. Materials and methods

2.1 Materials

Luria-Bertani (LB) broth, LB broth with agar and analytical grade paraffin oil were supplied by Sigma Aldrich, UK. The other oils used in this study (vegetable, olive and corn) were commercially sourced. Polyglycerol polyricinoleate (PGPR) was provided by Palsgaard, Denmark and SHRO was supplied by Matoke Holdings, UK. All materials were used as delivered and no further purification or modifications were made.

2.2 Emulsion formulation

Emulsions were created in a two-step process using a T18 Ultra Turrax® disperser (IKA, UK). The oil phase was combined with PGPR for 1 min at 10,000 rpm. SHRO was then added dropwise, using a 5 mL syringe, to the oil and surfactant mixture. After the SHRO was added, shear was applied at 10,000 rpm until a total time of 10 minutes had elapsed. The final emulsions were stored in a temperature controlled laboratory set at 21°C until needed.

Unless otherwise indicated, percentages are denoted as (v/v) and indicate the amount of SHRO present in the formulation. The oil used can be assumed to be paraffin oil with the surfactant volume set at 2% unless otherwise stated.

2.3 Visual characterisation of emulsion stability

Emulsions were formulated according to the method in section 2.1 and monitored over a 7-day period at 21°C. At each time point (1, 2, 3 and 7 days) an image was taken of each individual sample. To enable measurement calibration a fixed scale was placed next to the sample during capture. Images were visually assessed to determine if droplet sedimentation or separation of phases occurred. *Image J* software (1.47v National Institutes of Health, USA) was used to calibrate images and obtain a quantitative measurement of separation.

2.4 Rheological characterisation of SHRO in paraffin oil emulsions

An AR-G2 rheometer (TA Instruments, UK) with sandblasted parallel plates (size = 40mm, gap height = 1mm) was used to determine the viscoelastic properties of emulsions immediately after manufacture (day 0) and after 7 days of storage at 21°C. Prior to testing on day 7 the emulsions were shaken for 1 minute by hand in order to resuspend the droplets. Viscosity was determined by means of a shear rate sweep from 1.0 to 100.0 s⁻¹ conducted over a period of 5 minutes at 21°C. From this sweep, a shear rate of 4.1 s⁻¹ was used to characterise behaviour prior to storage. This shear rate is representative of a low shear post-mixing state such as that achieved following a shake before use directive [25]. A shear rate of 99.7 s⁻¹ was isolated to describe the behaviour under higher shear application such as extrusion from a tube [26].

The linear viscoelastic region (LVR) was identified for all tested emulsions using a strain sweep with frequency set at 1 Hz. This data was used to select a percentage strain value which fell within the LVR. Frequency sweeps were then carried out from 0.1 to 100 Hz at 21°C and 0.5% strain to obtain storage (G') and loss (G'') moduli.

It was important to determine the rate of viscosity recovery after simulated application to ensure that the emulsion structure was not altered. Formulations were subjected to 100 s^{-1} for 30 seconds at 21°C to mimic extrusion. Shear rate was then reduced to 1.0 s^{-1} and viscosity monitored for 30 seconds. This process was repeated thrice, and the hysteresis results plotted.

2.5 Size analysis of dispersed SHRO droplets

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 Conductivity measurements

Conductivity was measured using a HI99300 conductivity test meter (Hanna Instruments, UK). Measurements were taken of individual components as well as both prior and post addition of deionised water and shear. Emulsions were diluted with deionised water in a 1:2 ratio and vortexed for 1 minute to trigger inversion.

2.7 Hydrogen peroxide release from SHRO emulsions

A fluorescent assay kit (Sigma Aldrich, UK) was used to determine hydrogen peroxide release from SHRO 30, 50 and 60 % emulsions. Prior to testing a calibration curve was created using hydrogen peroxide standards 10, 3, 1, 0.3, 0.1, 0.03, 0.01 and $0\mu\text{M}$. A further calibration curve was created using SHRO 2.0, 1.5, 1.0 and 0.5 g/L. This was used to determine the dilution factors used given the concentration limits of the assay. For the assay, 1 mL of each emulsion was diluted with 2 mL of deionised water and vortexed for 10 seconds. This was then further diluted by adding 0.1 mL to 30 mL of deionised water (1:300) at each time point (1, 3, 6, 12 and 24 hours). The manufacturer's instructions were followed, briefly the following standard solutions were made: a master mix containing 50 μL red peroxidase substrate, 200 μL of 20 units/mL peroxidase, 4.75 mL assay buffer, and hydrogen peroxide standards between $0\mu\text{M}$ and $10\mu\text{M}$.

50 μL of sample or standard was then added to each well and 50 μL of master mix was added. Wells were mixed by pipetting, protected from light and incubated at room temperature for 30 minutes. The fluorescence intensity was then measured ($\lambda_{\text{ex}} = 540\text{nm}$ / $\lambda_{\text{em}} = 590\text{nm}$) using a Tecan Spark plate reader (Tecan Trading AG, Switzerland). Each emulsion was tested in triplicate.

2.8 In-vitro efficacy of SHRO emulsions

LB broth and LB broth with agar was reconstituted with distilled water to concentrations of 20 g/L and 35 g/L respectively, both were sterilised by autoclaving for 20 minutes at 121°C and 100 KPa.

Overnight cultures of *Staphylococcus aureus* (*S. aureus* - ATCC 29213), *Escherichia coli* (*E. coli* - MG1655) and *Pseudomonas aeruginosa* (*P. aeruginosa* - NCTC 13437) were prepared by inoculating one colony of each strain into 5 mL of LB broth. The optical density of each overnight culture was

then measured at 600 nm using a Spectronic Helios Gamma UV-Vis Spectrophotometer (Thermo Fisher Scientific, UK) and diluted to 0.04.

Agar plates were created from LB broth with agar and inoculated with bacteria. A 10 mm sterile hole borer was then used to create a well in the center of the inoculated agar. 1 mL of each emulsion was diluted with 2 mL of deionised water and vortexed for 1 minute in order to trigger the release of ROS. 250 μ L of diluted emulsion was then added to the well and incubated at 37°C for 24 hours. Zones of inhibited bacterial growth were then measured, and the bore hole diameter was deducted from the total zone size. Each emulsion formulation and bacterial strain were run in triplicate.

2.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism® 5.0 software. Two-tailed Welch's t-tests were used to determine statistical significance. Values of $p < 0.05$ were considered significant. Data is presented as mean $\pm$ standard deviation.

3. Results

3.1 Stability of SHRO emulsion

Paraffin oil emulsions containing 30% SHRO and 2% PGPR were found to have a slower sedimentation rate ($29.6 \pm 8.6\%$) over 7 days than emulsions formulated with olive ($73.9 \pm 1.1\%$), corn ($86.0 \pm 4.7\%$) or vegetable oil ($78.5 \pm 1.1\%$) (Figure 2a). With the exception of paraffin oil, the resultant emulsion instabilities were not reversible by inversion or shaking as phase separation had occurred. Emulsions comprised of paraffin oil maintained the integrity of the SHRO droplets and after initial sedimentation could be re-dispersed by inversion. Coalescence and phase separation did not occur in paraffin oil emulsion systems over the storage periods investigated.

An increase in SHRO concentration for paraffin oil samples resulted in a lower rate of sedimentation. Notably, emulsions containing $>50\%$ SHRO showed no indication of sedimentation over a 7 day period. Emulsions containing 40 and 30% SHRO displayed sedimentation rates of 9.0 ± 1.8 and $29.6 \pm 8.6\%$, respectively over 7 days (Figure 2b).

Further testing determined that emulsions containing $< 2\%$ PGPR sedimented after 7 days. After 24 hours sedimentation was observed for both the 60 and 50% SHRO emulsions, which contained 0.5% PGPR with sedimentation rates of 15.3 ± 1.0 and $22.8 \pm 5.0\%$, respectively. Sedimentation progressed and at day 7 was recorded for 60% SHRO emulsions at 40.6 ± 2.0 and $50.4 \pm 5.0\%$ for 50% SHRO emulsions (Figure 2c). With an increase in surfactant to 1%, sedimentation was reduced. After 3 days emulsions containing 60% SHRO still displayed no sedimentation, emulsions that contained 50% SHRO, however sedimented by $3.2 \pm 1.1\%$. After 7 days, 60 and 50% SHRO emulsions containing 1% PGPR showed no significant difference in sedimentation with rates of 13.1 ± 0.3 and $13.5 \pm 1.1\%$ respectively (Figure 2c). For its improved stability 2% PGPR was used for the remainder of the study.

Figure 2. Rate of droplet sedimentation as a function of the formulation undertaken at room temperature: oil type (a), dispersed phase (SHRO) concentration (b) and emulsifier (PGPR) concentration (c).

3.2 SHRO emulsion rheology

The ratio of SHRO to paraffin oil within the emulsion significantly alters the overall viscosity (Figure 3a). An increase in the concentration of SHRO increased viscosity across all shear rates tested ($1.0 - 100 \text{ s}^{-1}$). Apparent viscosities on day 0 and day 7 respectively measured at $19.3 \pm 0.2 \text{ Pa.s}$ and $13.42 \pm 0.2 \text{ Pa.s}$ (60%), $9.7 \pm 0.3 \text{ Pa.s}$ and $6.9 \pm 0.5 \text{ Pa.s}$ (50%) and $1.4 \pm 0.2 \text{ Pa.s}$ and $1.8 \pm 0.7 \text{ Pa.s}$ (30%) at a shear rate of 4.1 s^{-1} . After storage for 7 days at 21°C there was a 20 – 35% reduction in viscosity at all tested shear rates between 60 and 50% SHRO emulsions. In contrast, the 30% SHRO emulsion formulation showed no significant difference in viscosity after the 7-day storage period (Figure 3b).

The apparent viscosity profile of the emulsions differed to that of the control, unprocessed SHRO. At lower shear (4.1 s^{-1}), 60 and 50% SHRO emulsions had a higher viscosity than that of SHRO alone. In contrast, at a higher shear rate (99.7 s^{-1}), it was found that all of the emulsions except 60% SHRO, displayed lower apparent viscosities on day 0 compared to SHRO alone (Figure 3c).

When SHRO emulsions are rapidly switched from a low (1.0 s^{-1}) to a higher shear (100 s^{-1}) the viscosity reduces but quickly recovers exhibiting shear thinning behaviour (Figure 3d). The SHRO

emulsions did display hysteresis upon application and removal of shear as the recovery viscosity of the formulation is lower than that of the initial measurement (Figure 3d).

Analysis of the viscoelastic properties of the emulsions determined that higher concentrations of SHRO exhibited higher G' and G'' values. At 5 Hz, for 60 and 50% SHRO emulsions, the G' was higher than that of the G'' . However, 30% SHRO emulsions exhibited similar G' and G'' values at 5 Hz. At higher frequencies (30 Hz) G'' exceeded G' for all formulations. The frequency at which crossover occurred was found increased with increasing SHRO concentration. At day 0 this phenomenon occurred at 63.1 Hz, 50.1 Hz and 1.6 Hz for 60, 50 and 30% SHRO emulsions, respectively and 50.1 Hz, 39.8 Hz and 0.8 Hz on day 7 (Figure 3f).

Figure 3. Visualisation of 60, 50 and 30% SHRO emulsion formulations (a), rheological characterisation of 60, 50 and 30% SHRO emulsions immediately after formulation (day 0) and after 7 days of storage at room temperature highlighting apparent viscosity (b), the viscosity of 60, 50 and 30% SHRO emulsions normalised against SHRO at low (4.1 s^{-1}) and high (99.7 s^{-1}) shear (c), shear thinning behaviour of 60, 50 and 30% SHRO emulsions at shear rates of 1.0 s^{-1} and 100 s^{-1} conducted at 21°C (d), frequency sweep of 60 and 30% SHRO emulsions conducted at 0.5% strain at 21°C (e) and the frequency at which G'' is greater than that of G' for 60, 50 and 30% SHRO emulsions, conducted at 0.5% strain at 21°C (f). $n = 3$ and error bars represent standard deviation.

3.3 SHRO emulsion characterisation

The size of the dispersed SHRO droplets were investigated after manufacture (day 0) and following 7 days of storage. The median diameter (D_{50}) of the droplets at day 0 for 30, 50 and 60% SHRO emulsions were found to be $4.92 \pm 0.44 \text{ }\mu\text{m}$, $2.09 \pm 0.07 \text{ }\mu\text{m}$ and $2.07 \pm 0.02 \text{ }\mu\text{m}$, respectively. For all samples there was no significant difference found in D_{50} droplet size between days 0 and day 7 (Table 1).

Table 1. Droplet sizes in 60, 50 and 30% SHRO emulsions at days 0 and 7. A two tailed t-test was conducted between D_{50} measurements for days 0 and day 7 in order to assess significance ($P < 0.05$). $n=3$, $\pm$ standard deviation. *N=No and Y=Yes

Emulsion	Size (μm)			Sig. Diff. P < 0.05 (Y/N)*
	D ₁₀	D ₅₀	D ₉₀	
30% SHRO				
Day 0	1.70	4.92	11.77	N
	± 0.39	± 0.44	± 1.48	
Day 7	1.48	4.11	9.88	
	± 0.21	± 0.31	± 0.64	
50% SHRO				
Day 0	1.22	2.09	3.61	N
	± 0.08	± 0.07	± 0.09	
Day 7	1.23	2.09	3.48	
	± 0.03	± 0.02	± 0.01	
60% SHRO				
Day 0	1.18	2.07	41.89	N
	± 0.12	± 0.02	± 54.4	
Day 7	1.12	2.42	72.13	
	± 0.09	± 0.26	± 5.93	

In order to assess the inversion of the emulsion under application of water and shear, conductivity measurements were taken. Neat paraffin oil has no measurable conductivity ($0\ \mu\text{S}$) as such when it is the continuous phase the emulsions, regardless to SHRO concentration, exhibit no conductivity. Following addition of deionised water and applied shear, conductivity measurements showed readings of $219.7\pm6.7\ \mu\text{S}$, $204.7\pm6.7\ \mu\text{S}$ and $181.3\pm8.4\ \mu\text{S}$ for 60, 50 and 30% SHRO emulsions respectively, indicating CPI (Figure 4a).

The ability to maintain antimicrobial efficacy of SHRO once formulated into an emulsion is paramount. Therefore, it was important to assess the levels of hydrogen peroxide production following phase inversion of the SHRO emulsions. It was found that the emulsion with the highest ratio of SHRO produced the most hydrogen peroxide; 60% SHRO emulsions produced $595.1\pm22.1\ \mu\text{M/mL}$ after 24 hours and the 50 and 30% formulations releasing less at $249.9\pm3.9\ \mu\text{M}$ and $83.5\pm3.0\ \mu\text{M}$ respectively (Figure 4b).

Figure 4. Conductivity of SHRO emulsions before and after phase inversion (a) and the release of hydrogen peroxide over time (b). $n = 3$ and error bars represent standard deviation.

3.4 Antimicrobial efficacy

Unprocessed SHRO controls showed that the bioengineered honey had the potential to be efficacious against *S. aureus*, *P. aeruginosa* and *E. coli* (Figure 5a). Emulsions with 60, 50 and 30% SHRO were then tested against the same bacterial strains with efficacy compared to neat SHRO diluted in water to the same concentration as would be found within each emulsion. All formulations were shown to inhibit the growth of the Gram-positive bacteria, *S. aureus* (Figure 5b). However, only 60 and 50% SHRO emulsions produced enough ROS to inhibit the growth of Gram-negative bacteria *P. aeruginosa* and *E. coli* (Figures 5c and d). No significant difference was identified between the efficacy of the emulsions and their equivalent, neat, SHRO dilution when tested against *S. aureus*. These results were replicated in both Gram-negative bacteria with the exception of the 30% SHRO emulsions which did not inhibit bacterial growth (Figure 5).

Figure 5. SHRO treated *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* (a), Inverted SHRO emulsions and the zones of inhibition by treating *Staphylococcus aureus* (b), *Escherichia coli* (c) and *Pseudomonas aeruginosa* (d). Agar plates were inoculated with bacterial culture (OD_{600} 0.04) at 37°C for 24 hours before treatment. Bore hole size = 10mm.

4. Discussion

SHRO is engineered to deliver tailored doses of ROS and does not rely on specific flora or environmental conditions for its antibiotic activity like natural honeys, such as Manuka. Since it is engineered, this means that the level of potency can be controlled. Previous studies looking at the efficacy of SHRO have had promising results, but despite this, there are limitations associated with delivering it to wound sites [13–17,27]. Currently much like commercial honey, it is a concentrated polymer blend which is sticky, viscous and hard to handle. These characteristics make it difficult to apply. This study has demonstrated a method by which to deliver SHRO that enables stable storage whilst maintaining the ability to inhibit bacterial growth when ROS production is triggered. This is achieved by the formulation of emulsions, which undergo CPI when exposed to water and shear.

By creating a SHRO-in-oil emulsion it allows modification of the viscosity, a critical characteristic that may determine how a product may be delivered. In addition, by creating an emulsion devoid of free water there is no premature production of ROS. This is essential for efficacy and shelf life. One of the main challenges when developing an emulsion is addressing the inherent thermodynamic instability of the system. Investigation of SHRO stability within different oil phases showed that paraffin oil

produced an emulsion that did not coalesce or show signs of flocculation or creaming. Furthermore, the dispersed SHRO was found to sediment at a slower rate than emulsions formulated with olive, corn or vegetable oil (Figure 2a). In the cases of the emulsions containing vegetable, olive or corn oil the resultant instability was not reversible by means of inversion and shaking as phase separation had occurred. Although sedimentation is not ideal for this application, since it did not occur immediately and the dispersed droplets could be redistributed easily in paraffin oil, further investigation was deemed necessary. Notably, the need for redistribution of dispersed phases before application is common practise among formulations that contain unstable components. Shaking ensures an even distribution in droplets and therefore administration of a uniform dose, a factor highlighted for its importance by Stringer and Bryant in the formation of corticosteroid emulsions [28]. Promisingly, a comparison of droplet size between days 0 and 7 demonstrated that no coalescence occurred for any of the SHRO paraffin oil emulsions studied (Table 1).

Optimising dispersed phase ratios (30-60%) with a set concentration of PGPR (2%), found that formulations with a higher concentration of SHRO sedimented the least (Figure 2b). Further, no significant difference in droplet size for the 60% SHRO emulsion was observed over 7 days of storage. This effect is likely due to two factors. Firstly, the droplets formed in the 50 and 60% emulsions are small (around 2 μm) and suffer less from sedimentation compared to the 5 μm droplets of the 30% emulsion, thus reducing sedimentation at higher phase volumes [29]. The second factor is that as more SHRO is added the volume occupied by droplets in the system also increases. This higher packing fraction physically prevents sedimentation and results in an increase in viscosity (Figure 3), which is known to reduce sedimentation speed [30,31]. Notably, both 50 and 60% SHRO emulsions showed no sedimentation over a period of 7 days. The low HLB value (1.5) associated with PGPR clearly aids the stabilisation of the SHRO in oil system greatly. It was determined that 2% was the minimum concentration of PGPR required to stabilise these systems (Figure 2c).

Promisingly, all of the paraffin oil emulsions displayed shear thinning behaviour (Figure 3b). This will help to ease delivery, as common methods of administration, such as sprays and creams, often involve the application of shear. After storage for 7 days there was a significant ($p < 0.05$) reduction in viscosity at all tested shear rates for 60 and 50% SHRO, interestingly a phenomenon not apparent in the 30% formulations. The difference between the apparent viscosity of 60 and 50% SHRO emulsions on days 0 and 7 is likely due to an interaction between SHRO droplets, which requires greater shear than that exerted from shaking to mix the emulsion back to its original form [29,30]. This was not the case with the 30% SHRO emulsion; with greater range of movement, due to the lower packing fraction and lower interactions between droplets, viscosities comparable with that of day 0 could be achieved. As demonstrated by the results here and as described by Stokes law, an increase in viscosity slows the rate of sedimentation [30,32,33]. Future applications could use modifiers to increase the viscosity thus avoiding the need to shake before use. This may however prevent the use of delivery systems only suitable for lower viscosity fluids, for example pump spray bottles. This data highlights a trade-off between stability and ease of application; while the 30% emulsion exhibits $29.6 \pm 8.6\%$ sedimentation after 7 days it has the lowest viscosity widening the possible administration devices that could be used. That said, it is notable that under high shear all SHRO emulsions exhibited significantly reduced viscosities (Figure 3c), which will aid delivery and application. Further, it is advantageous that when all formulations were rapidly switched from high

to low shear, in the absence of water, viscosity is quickly recovered (Figure 3d). This means that the viscosity can be reduced in order to deliver the emulsion but then as the shear rate reduces the emulsion quickly becomes more viscous meaning it is more likely to be retained at the site of delivery [34,35]. The SHRO emulsions did display hysteresis, however, since this effect was minimal it is not deemed to affect the final application but warrants further investigation.

Frequency sweeps were also conducted to elucidate the viscoelastic properties and stability of the emulsions. Oscillatory rheology was used to determine the value of the storage (G') and loss modulus (G'') at low frequency values. This was concluded to be an important assessment of emulsion stability by Tadros, 2004 [31]. The viscosity profile of the emulsions revealed that both G' and G'' rapidly increase with frequency (Figure 3e), with the dependence on frequency considered to be due to the effect of energy dissipation within droplets [36]. The relative values of G' and G'' are known to be indicative of a change in fluid structure [37]. At 5 Hz, 60 and 50% SHRO emulsions exhibited a higher G' than G'' , which demonstrates they have a greater gel like structure. In contrast, 30% SHRO formulations were observed to have similar G' and G'' values ($28.9 \pm 8.7 - 54.0 \pm 17.1$ Pa) at this frequency. This correlates with observations that the stability is increased in emulsion formulations with higher concentrations of SHRO as droplets begin to structure and cause an increase in viscosity [38]. At higher frequencies the emulsions undergo a viscoelastic transition, this is where G'' exceeds that of G' [39]. This occurred for all samples tested indicating that the droplets within the emulsion interact at a small length scale and as such does not relax quickly. This decreases the elastic behaviour and thus becomes more viscous, or liquid-like [40]. The frequency at which the G' and G'' crossover increased with rising SHRO concentration. This means that the ease of delivery decreases with increased amount of SHRO as more energy is required to extrude the emulsion from the delivery device.

In addition to easing the delivery of SHRO, this work aimed to create an emulsion that could be triggered to release ROS *in-situ*. Through the addition of water under shear it was possible to cause the developed emulsions to catastrophically phase invert (CPI) leading to activation of SHRO. It is proposed that water may either be co-delivered with the emulsion or media at the wound site could be used in order to trigger release. However, since available moisture will significantly vary at different wound sites it is suggested that co-delivery may be a more reproducible approach [41,42]. In order to confirm that CPI had occurred, conductivity measurements were taken (Figure 4a). Neat paraffin oil has no measurable conductivity. When SHRO is dissolved in deionised water a current is allowed to flow due to the greater mobility of free ions [39]. Water was added to the emulsion and shear was applied, an increase in conductivity was measured ($181.3 \pm 14.6 - 219.7 \pm 6.7$ μ S) with formulations containing higher concentrations of SHRO allowing a greater current to flow. These measurements confirmed that CPI had occurred and that the continuous phase is a combination of SHRO and water.

It is known that the release of ROS, such as hydrogen peroxide is the dominant factor to the antimicrobial efficacy of SHRO [27]. As such, it was critical that SHRO droplets within the emulsion could still be activated to produce ROS after being exposed to water through phase inversion. As expected, it was found that the emulsion with the highest ratio of SHRO (60%) produced the most hydrogen peroxide; 595.0 ± 22.1 μ M after 24 hours. A controlled and predictable release is critical to ensuring an effective and safe dose is delivered throughout the period of application [43].

Promisingly, release of ROS was observed for the entire time period tested (Figure 4b). For a topical application, stable release over 24 hours would mean that the treatment would only have to be applied at most once daily and therefore makes it more convenient for the patient or healthcare professional applying the product.

Having assessed the viability of ROS production from the formulated SHRO emulsions, clinically relevant bacterial species found in topical wounds [44] were inoculated onto agar plates and the SHRO emulsions ability to inhibit growth was measured. Unprocessed SHRO controls showed that the bioengineered honey had the potential to be efficacious against *S. aureus*, *P. aeruginosa* and *E. coli* (Figure 5a). This is consistent with previous *in-vitro* evaluations of SHRO [13–16,27,45,46]. Despite releasing different levels of ROS over 24 hours, inhibition of Gram-positive bacteria *S. aureus* was achieved for all tested inverted emulsions (Figure 5b). In contrast, inhibition of *P. aeruginosa* and *E. coli* was only observed for emulsions containing 50 or 60% SHRO. It is known that Gram-negative bacteria are inherently harder to kill as compared with Gram-positive species they contain an extra layer of protection in the form of an outer membrane. Previous studies have demonstrated that higher doses of ROS are necessary to inhibit Gram-negative bacterial growth [47]. The ability to increase the baseline efficacy of SHRO, as reported by Dryden et al., [13] may provide a solution. Working with a batch of SHRO with higher ROS production would enable the lower viscosity characteristics of 30% SHRO emulsions to be exploited to ease application while delivering a dose also capable of killing Gram-negative bacteria.

5. Conclusion

In conclusion this study addresses the need for alternative topical infection treatments to current antibiotics. This is achieved by the successful formulation of an emulsion containing SurgihoneyRO™ (SHRO), which has been engineered to enable triggered release of antimicrobial reactive oxygen species via phase inversion. The hypothesis that an oil-based emulsion would protect, store, and release SHRO under shear and dilution with water at the time of application has been demonstrated *in-vitro*. This mechanism could be initiated by added or topical moisture, such as wound exudate and shear produced by hand or a delivery device.

Emulsion composition was optimised to achieve the desired shear thinning behaviour, easing delivery and promoting retention of the active at the site of infection. Stability observations revealed that a small degree of sedimentation did occur, over 7 days, for emulsions containing a dispersed phase of <50% SHRO, but this was shown to be reversible by shaking. Conveniently this redistributed droplets evenly, which is known to improve dose reproducibility. Hydrogen peroxide measurements indicated that formulations with higher concentrations of SHRO produced greater levels of reactive oxygen species. Emulsions with 30, 50, and 60% SHRO were tested against clinically relevant bacterial species; *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*. It was found that 60 and 50% SHRO emulsions were efficacious against all species. In summary, it is demonstrated that emulsion technologies can be used to effectively store and deliver water sensitive products, with triggered release mechanisms to actively combat clinically relevant infections.

Conflict of Interest: The authors declare no conflict of interest.

Acknowledgements: This research was supported by the EPSRC (Project number 1823302 – novel antimicrobial delivery systems) and undertaken in collaboration with Matoke Holdings Ltd.

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Highlights:

- Delivery of reactive oxygen species produced by a mechanism in engineered honey
- Formulation of an oil continuous emulsion to increase ease and control of delivery
- Triggered release of active when exposed to water and shear
- Demonstrates the ability to inhibit growth of clinically relevant bacteria

ACCEPTED MANUSCRIPT

a)

Oxidation of Glucose Mediated by Glucose Oxidase to Form Hydrogen Peroxide

b)

Paraffin Oil
+
PGPR
+
SurgihoneyRO™
(SHRO)

c) Emulsion Formed

+ H₂O and Shear

SHRO Continuous

d) Water Mixed

e) Phase Inversion

H₂O₂ Produced

Figure 1

Figure 2

Figure 3

a) **b)**

Figure 4

Figure 5