

A COMPOSITION, A METHOD OF MAKING THE SAME AND ITS USES THEREOF**CROSS-REFERENCE TO RELATED APPLICATIONS**

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This application claims priority to Singapore application number 1020211225V filed with the Intellectual Property Office of Singapore on 8 October 2021, the contents of which is hereby incorporated by reference in its entirety for all purposes.

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FIELD OF THE INVENTION

[0001] The present disclosure relates generally to a composition. The present disclosure also relates to a method of forming the composition, a kit comprising the composition, and a method of delivering an active agent to a target site. The present disclosure also relates to a method of treating a disease in a patient and medical uses of the composition.

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BACKGROUND OF THE INVENTION

[0002] Osteoarthritis (OA) affects 250 million people worldwide and is the most common form of chronic joint disease. It is characterized by pain, inflammation and degradation of multiple joint tissues. As the disease progresses, there will be degradation of the cartilage, degeneration of menisci and ligaments, and subchondral bone erosion with osteophyte formation. While the disease etiology remains elusive, it is increasingly recognized that inflammation plays a central role in OA pathogenesis and disease progression. Local release of inflammatory mediators by local joint tissues including cartilage, subchondral bone and synovium are known to contribute to joint pain and degradation in OA. Current treatments for OA are mainly symptomatic therapies using acetaminophen, non-steroidal anti-inflammatory drugs (NSAIDs) and opioids. While effective to a certain extent in managing pain, swelling and inflammation, these therapies have limited to no effects on joint repair and may cause undesirable side effects and tolerance issues. In the effort to improve the efficacy and reduce the side effects of NSAIDs, topical formulations have been developed for the dermal delivery of a few anti-inflammatory agents to the joint. Although topical formulations were developed to deliver NSAIDs through the skin, these anti-inflammatory agents

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were reported to cause dermal side effects such as rash, dry skin, dermatitis or urticaria. Moreover, the use of topical NSAIDs gels or creams to treat pain has been reported to cause a photo-induced contact dermatitis. Hence, there is the compelling need to consider other anti-inflammatory agents as alternative option.

5 [0003] Natural products are frequently used in Traditional Chinese Medicine (TCM) to reduce “heat”, and were reported to have chondroprotective effects when it was administered into rat OA model via intra-articular injection. This may suggest that its efficacy can be comparable to the existing pharmacotherapy in modulating inflammation in osteoarthritis. The proposed mechanism of these natural compounds includes mainly the inhibition of interleukin (IL)-6 proinflammatory
10 cytokine. This proinflammatory cytokine is reported to be one of the key players in the pathophysiology of OA, and these natural compounds can potentially be applied as an adjuvant therapy for OA. While these natural products had demonstrated promising efficacy in modulating inflammation in OA, it suffers from limited bioavailability due to poor absorption and extensive metabolism in liver when taken orally. Intra-articular injection may circumvent the limited
15 bioavailability, but this procedure is invasive and can only be carried out by physicians in the clinical settings, which can lead to poor patient compliance.

[0004] When considering alternatives to intra-articular injections, topical administration is a possible route of administration due to its non-invasive nature and ease of application. In order to deliver therapeutics through the skin, a major challenge is the presence of the stratum corneum
20 (SC). The SC is the outermost layer of the epidermis and consists of densely packed and highly keratinized dead cells. They act as a rate-limiting barrier for molecular delivery. Given the structure of the SC, various dermal delivery systems have been explored, with the aim of increasing the skin permeability and facilitate the delivery of bioactive molecules. Among the dermal dosage forms, niosomes offer great promise in drug delivery. Comprising non-ionic surfactants that are
25 known as chemical penetration enhancers, these chemicals organize themselves into lipid bilayers and hydrophilic core vesicles that promote passage of active molecules by affecting the fluidity of the SC. Furthermore, niosomes were originally formulated for cosmetic applications, providing evidence for dermal delivery. However, due to the physical instability of niosomes, these vesicles are formulated as proniosomes that exist in a dehydrated form, with the niosomes in the form of
30 vesiculating lamella stacking while forming the gel.

[0005] The mechanism of the proniosome gel formation using the coacervate separation method was proposed earlier. Niosomes are packed in a lamellar liquid crystalline structure and

further absorption of water from the skin surface leads to swelling and release of the niosomes from the formulation (see Fig. 1).

[0006] Given the potential of the proniosome gel to be used in dermal applications, a few studies have demonstrated the ability of the formulation in delivering their encapsulated compounds topically. However, conventional encapsulation strategies rely heavily on screening different types of non-ionic surfactants to achieve a high drug loading efficiency and to increase the concentration of the drug delivered through the skin. The encapsulation efficiency of the formulation is dependent on the physicochemical properties of the drug, and this may not be a feasible approach in formulating the proniosome gel. Furthermore, the type and composition of the non-ionic surfactants in the formulation may affect the gelation ability of the formulation and this may impact the delivery efficacy of the drug through the skin.

[0007] Accordingly, there is a need to investigate the mechanism of the proniosome gel and to prepare a formulation based on the mechanism of the proniosome gel to enhance the dermal delivery of drugs.

[0008] There is a need to provide a method of forming the composition that has the features above. There is a need to provide uses of the composition that ameliorates the problems described above.

SUMMARY OF THE INVENTION

[0009] In one aspect, the present disclosure relates to a composition comprising: a) a first surfactant having a transition temperature of at least 50 °C; b) a second surfactant having a transition temperature of less than 20 °C; c) a third surfactant having a hydrophilic-lipophilic balance (HLB) value of at least 15; d) cholesterol; e) an active agent; f) a first solvent; and g) a second solvent.

[0010] Advantageously, by having the first surfactant with a transition temperature of at least 50 °C and the second surfactant with a transition temperature of less than 20 °C (and in some examples, the third surfactant with a hydrophilic-lipophilic balance (HLB) value of at least 15), this may influence the release profile of the active agent, allow one to tailor the drug release profile, enhance the pharmacological efficacy of the compound and/or improve the desired clinical outcomes from the dermal application.

[0011] Advantageously, the composition may have a reduced stiffness due to the combination of the surfactants having different transition temperatures.

[0012] Further advantageously, the composition may have an improved ability to absorb water due to the combination of the surfactants having different HLB values.

[0013] Still further advantageously, the composition having a reduced stiffness and an improved ability to absorb water may be more effectively applied in medical uses.

5 [0014] In another aspect, the present disclosure relates to a method of forming a composition comprising the steps of: (a) heating a first surfactant, a second surfactant, a third surfactant, cholesterol, an active agent and a first solvent to form a mixture; and (b) heating the mixture of step (a) with a second solvent and cooling the mixture to form the composition, wherein the first surfactant has a transition temperature of at least 50 °C, the second surfactant has a transition
10 temperature of less than 20 °C, and the third surfactant has a hydrophilic-lipophilic balance (HLB) value of at least 15.

[0015] In another aspect, the present disclosure relates to a kit comprising the composition as described herein and instructions for using the composition as described herein.

[0016] The present disclosure relates to a method of treating a disease in a patient, comprising
15 administering to the patient an effective amount of the composition as described herein, wherein the disease is selected from the group consisting of inflammation, cancer, infection and combinations thereof.

[0017] The present disclosure also relates to a method of delivering an active agent to a target site, comprising the steps of:

20 (a) providing a composition comprising the active agent as described herein or as formed according to the method as described herein; and

(b) administering the composition at a first site to allow the active agent to move to or be transported to the target site.

[0017a] In another aspect, the present disclosure relates to an *in vitro* method of delivering an
25 active agent to a target site, comprising the steps of:

(a) providing a composition comprising the active agent as described herein or as formed according to the method as described herein; and

(b) administering the composition at a first site to allow the active agent to move to or be transported to the target site.

30 [0018] Advantageously, the composition can be administered to a subject via topical administration or dermal administration. The composition can be used to administer the active agent to a subject with a disease (such as osteoarthritis (OA)), allowing local accumulation of the active agent while avoiding their systemic side effects.

[0019] Advantageously, the combination of surfactants with different physical properties may aid to facilitate spreading of the composition (in a gel form) onto a subject's skin, enhancing the release of the active agent from the composition and thereby increasing increase the amount of active agent delivered through the skin.

- 5 [0020] In another aspect, the present disclosure relates to the composition as described herein for use in therapy.

[0021] In another aspect, the present disclosure relates to the composition as described herein for use in treating or preventing a disease selected from the group consisting of inflammation, cancer, infection or combinations thereof.

[0022] In another aspect, the present disclosure relates to the use of the composition as described herein in the manufacture of a medicament for the treatment or prevention of a disease selected from the group consisting of inflammation, cancer, infection or combinations thereof.

DEFINITIONS

[0023] The term “surfactant” as used herein refers to substances comprising hydrophilic moieties and lipophilic moieties. Therefore, these substances are amphiphilic.

[0024] The term “non-ionic surfactant” as used herein refers to substances wherein all hydrophilic and lipophilic moieties are covalently linked to each other. Therefore, they do not comprise cations or anions.

[0025] The term “niosome” as used herein refers to a vesicle comprising a shell of non-ionic surfactants and a cavity filled with an aqueous medium. The vesicles may further comprise cholesterol in the shell. The vesicles may further comprise an active agent that may be in the shell or in the cavity.

[0026] The term “proniosome” as used herein refers to a composition that consists of niosomes that are compacted together to form a lamellar liquid crystal due to the lack of the aqueous medium. Niosomes are released upon hydration with an aqueous medium.

[0027] The term “transition temperature” when used to define a solid substance refers to the temperature at which the substance converts from an elastic solid-like behaviour into a viscous liquid.

[0028] The term “phosphate buffered saline” as used herein refers to a solution comprising water, disodium hydrogen phosphate, sodium chloride, and optionally potassium chloride or potassium dihydrogen phosphate. The solution may have a pH value in the range of about 6 to 8. The solution may be used as a solvent, optionally in combination with other solvents, to dissolve substances.

[0029] The term “hydrophilic-lipophilic balance value” as used herein refers to a property of a substance that is derived from molar masses of hydrophilic and lipophilic moieties present in the substance. It is calculated as $20 \times M_h/M$, wherein M_h is the total molar mass of the hydrophilic moieties and M is the total molar mass of the substance.

[0030] Unless specified otherwise, the terms “comprising” and “comprise”, and grammatical variants thereof, are intended to represent “open” or “inclusive” language such that they include recited elements but also permit inclusion of additional, unrecited elements.

[0031] As used herein, the term “about”, in the context of concentrations of components of the formulations, typically means +/- 5% of the stated value, more typically +/- 4% of the stated value, more typically +/- 3% of the stated value, more typically, +/- 2% of the stated value, even more typically +/- 1% of the stated value, and even more typically +/- 0.5% of the stated value.

[0032] Throughout this disclosure, certain embodiments may be disclosed in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the disclosed ranges. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub-ranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed sub-ranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

DETAILED DESCRIPTION OF OPTIONAL EMBODIMENTS

[0033] Exemplary, non-limiting embodiments of a composition will now be disclosed. The composition comprises: a) a first surfactant having a transition temperature of at least 50 °C; b) a second surfactant having a transition temperature of less than 20 °C; c) a third surfactant having a hydrophilic-lipophilic balance (HLB) value of at least 15; d) cholesterol; e) an active agent; f) a first solvent; and g) a second solvent.

[0034] The composition may comprise: a) a first surfactant having a transition temperature of at least 50 °C; b) a second surfactant having a transition temperature of less than 20 °C; c) a third surfactant having a hydrophilic-lipophilic balance (HLB) value of at least 15; d) cholesterol; e) an active agent; f) a first solvent; and g) a second solvent.

[0035] The composition may consist essentially of: a) a first surfactant having a transition temperature of at least 50 °C; b) a second surfactant having a transition temperature of less than 20 °C; c) a third surfactant having a hydrophilic-lipophilic balance (HLB) value of at least 15; d) cholesterol; e) an active agent; f) a first solvent; and g) a second solvent.

[0036] In the composition, at least one of the first surfactant, the second surfactant or the third surfactant may be a non-ionic surfactant. At least two of the first surfactant, the second surfactant or the third surfactant may be a non-ionic surfactant. The first surfactant, the second surfactant and the third surfactant may be a non-ionic surfactant. In the case when two or all of the first, second and third surfactants are non-ionic, the type of non-ionic surfactant may be the same or different.

[0037] The composition may be a proniosome. The composition may be a proniosome gel and as such, may have a highly ordered gel structure with an increased mechanical strength which is important in order to maintain the stiffness of the formulation at ambient conditions. The proniosome gel self-assemble into rehydrated vesicles known as niosomes, which then carries or delivers the active agent to a desired site. The use of the surfactants in the composition (such as the proniosome gel) may aid to enhance permeability of the active agent through the skin and as it increases the therapeutic efficacy locally, it also results in the reduction of potential side effects arising from systemic administration.

[0038] Without being bounded by theory, the inventors believe that the physical properties of the surfactants (such as the non-ionic surfactant(s)) can affect the release profile of the active agent from the formulation. Additionally, by modulating the hydration ability of the composition, this may allow for the efficient release of the active agent from the composition with minimal mechanical force. At conventional skin surface temperature of 32 °C, the reduction of mechanical strength allows for the increased release of niosomes from the composition or formulation. Advantageously, when the G'' value (which is the loss modulus of the composition) is more than the G' value (which is the storage modulus of the composition), resulting in lower elasticity, the composition is able to behave predominantly more liquid and results in the ease of spreading the composition or formulation.

[0039] In the composition, the first surfactant may be sorbitan monostearate.

[0040] The first surfactant may have a weight percentage in the range of about 5.7 weight% to about 74.8 weight%, about 20 weight% to about 74.8 weight%, about 40 weight% to about 74.8 weight%, about 60 weight% to about 74.8 weight%, about 5.7 weight% to about 60 weight%, about 5.7 weight% to about 40 weight%, about 5.7 weight% to about 20 weight% or about 20 weight% to about 30 weight% based on the total weight of the composition.

[0041] The transition temperature of the first surfactant is at least about 50 °C, at least about 55 °C, at least about 60 °C, at least about 65 °C, at least about 70 °C, about 50 °C to about 70 °C, about 50 °C to about 65 °C, about 50 °C to about 60 °C, about 50 °C to about 55 °C, about 55 °C to about 70 °C, about 60 °C to about 70 °C, or about 65 °C to about 70 °C. In the composition, the

second surfactant may be sorbitan monooleate, sorbitan monolaurate or combinations thereof. The second surfactant may have a weight percentage in the range of about 2.8 weight% to about 72 weight%, about 20 weight% to about 72 weight%, about 40 weight% to about 72 weight%, about 60 weight% to about 72 weight%, about 2.8 weight% to about 60 weight%, about 2.8 weight% to about 40 weight%, about 2.8 weight% to about 20 weight% or about 10 weight% to about 15 weight% based on the total weight of the composition.

[0042] The transition temperature of the second surfactant is less than about 20 °C, less than about 15 °C, less than about 10 °C, less than about 5 °C, less than about 0 °C, less than about -5 °C, less than about -10 °C, less than about -15 °C, less than about -20 °C, about -20 °C to about 20 °C, about -20 °C to about 15 °C, about -20 °C to about 10 °C, about -20 °C to about 5 °C, about -20 °C to about 0 °C, about -20 °C to about -5 °C, about -20 °C to about -10 °C, about -20 °C to about -15 °C, about -15 °C to about 20 °C, about -15 °C to about 15 °C, about -15 °C to about 10 °C, about -15 °C to about 5 °C, about -15 °C to about 0 °C, about -15 °C to about -5 °C, about -15 °C to about -10 °C, about -10 °C to about 20 °C, about -10 °C to about 15 °C, about -10 °C to about 10 °C, about -10 °C to about 5 °C, about -10 °C to about 0 °C, about -10 °C to about -5 °C, about -5 °C to about 20 °C, about -5 °C to about 15 °C, about -5 °C to about 10 °C, about -5 °C to about 5 °C, about -5 °C to about 0 °C, about 5 °C to about 20 °C, about 5 °C to about 15 °C, about 5 °C to about 10 °C, about 10 °C to about 20 °C, about 10 °C to about 15 °C, or about 15 °C to about 20 °C.

[0043] The second surfactant may comprise or contain a cis-alkene functional group on the alkyl chain in the surfactant. By having this cis-alkene functional group, this may aid in enhancing permeation of the bilayer and thus improves gel spreadability and reduction of mechanical strength of the proniosome gel. Furthermore, the presence of the cis-alkene functional group on the alkyl chain corresponds to an increased critical strain value which results in reduced rigidity of the composition (when in the form of a proniosome gel) and allowed it to be more malleable under high shear strain. In the composition, the third surfactant may be polyoxyethylene (20) sorbitan monolaurate.

[0044] The third surfactant may have a weight percentage in the range of about 0.71 weight% to about 69.8 weight%, about 20 weight% to about 69.8 weight%, about 40 weight% to about 69.8 weight%, about 60 weight% to about 69.8 weight%, about 0.71 weight% to about 60 weight%, about 0.71 weight% to about 40 weight%, about 0.71 weight% to about 20 weight% or about 3 weight% to about 4 weight% based on the total weight of the composition.

[0045] The hydrophilic-lipophilic balance (HLB) value of the third surfactant is at least about 15, at least about 16, at least about 17, at least about 18, at least about 19, at least about 20, at least

about 15 to about 20, at least about 16 to about 20, at least about 17 to about 20, at least about 18 to about 20, at least about 19 to about 20, at least about 15 to about 19, at least about 16 to about 19, at least about 17 to about 19, at least about 18 to about 19, at least about 15 to about 18, at least about 16 to about 18, at least about 17 to about 18, at least about 15 to about 17, at least about 16 to about 17, at least about 15 to about 16. In the composition, the cholesterol may have a weight percentage in the range of about 0.74 weight% to about 69.8 weight%, about 20 weight% to about 69.8 weight%, about 40 weight% to about 69.8 weight%, about 60 weight% to about 69.8 weight%, about 0.74 weight% to about 60 weight%, about 0.74 weight% to about 40 weight%, about 0.74 weight% to about 20 weight% or about 3 weight% to about 4 weight% based on the total weight of the composition.

[0046] The combination of the first surfactant, the second surfactant, the third surfactant and cholesterol may have a combined hydrophilic-lipophilic balance value in the range of about 5 to 6, about 5 to 5.5 or about 5.5 to 6.

[0047] In the composition, the active agent may be a hydrophobic molecule.

[0048] The active agent may additionally or alternatively be a drug molecule. The active agent may be an anti-inflammatory agent, an anticancer agent or an anti-infection agent. The active agent may be berberine, capsaicin or a salt or a combination thereof.

[0049] The active agent may have a weight percentage in the range of about 0.9 weight% to about 70 weight%, about 20 weight% to about 70 weight%, about 40 weight% to about 70 weight%, about 60 weight% to about 70 weight%, about 0.9 weight% to about 60 weight%, about 0.9 weight% to about 40 weight%, about 0.9 weight% to about 20 weight% or about 0.5 weight% to about 1 weight% based on the total weight of the composition.

[0050] In the composition, the first solvent may be selected from the group consisting of methanol, ethanol, propanol, isopropanol, butanol, 1,2-propanediol, 1,3-propanediol or combinations thereof.

[0051] The first solvent may have a weight ratio in the range of about 10 weight% to about 79.1 weight%, about 20 weight% to about 79.1 weight%, about 40 weight% to about 79.1 weight%, about 60 weight% to about 79.1 weight%, about 10 weight% to about 60 weight%, about 10 weight% to about 40 weight%, about 10 weight% to about 20 weight% or about 15 weight% to about 20 weight% based on the total weight of the composition. In the composition, the second solvent may be selected from the group consisting of water, a phosphate buffered saline or combinations thereof.

[0052] The second solvent may have a weight ratio in the range of about 10 weight% to about 79.1 weight%, about 20 weight% to about 79.1 weight%, about 40 weight% to about 79.1 weight%, about 60 weight% to about 79.1 weight%, about 10 weight% to about 60 weight%, about 10 weight% to about 40 weight%, about 10 weight% to about 20 weight% or about 20 weight% to about 30 weight% based on the total weight of the composition. In the composition, the first solvent and the second solvent may be isopropanol and phosphate buffered saline respectively at a weight ratio in the range of about 1:1 to about 7:5.

[0053] In the composition, the first surfactant may have a weight percentage in the range of 5.7wt% to 74.8 wt%; the second surfactant may have a weight percentage in the range of 2.8 wt% to 72 wt%; the third surfactant may have a weight percentage in the range of 0.71 wt% to 69.8 wt%; the cholesterol may have a weight percentage in the range of 0.74 wt% to 69.8 wt%; the active agent may have a weight percentage in the range of 0.9 wt% to about 70 wt%; the first solvent may have a weight ratio in the range of 10 wt% to 79.1 wt%; or the second solvent may have a weight ratio in the range of 10 wt% to 79.1 wt%, based on the total weight of the composition.

[0054] In the composition, the first surfactant, the second surfactant, the third surfactant, cholesterol, the first solvent, the second solvent and the active agent may have a weight ratio of about 24.8 : 12.4 : 3.4 : 3.2 : 30.5 : 24.8 : 0.9.

[0055] In the composition, (a) the first surfactant is sorbitan monostearate; (b) the second surfactant is sorbitan monooleate, sorbitan mololaurate or combinations thereof; (c) the third surfactant is polyoxyethylene (20) sorbitan monolaurate; (d) cholesterol; (e) the active agent is a hydrophobic molecule; (f) the first solvent is selected from the group consisting of methanol, ethanol, propanol, isopropanol, butanol, 1,2-propanediol, 1,3-propanediol or combinations thereof; and/or (g) the second solvent is selected from the group consisting of water, a phosphate buffered saline or combinations thereof.

[0056] The composition may be formed by the method as described herein.

[0057] Exemplary, non-limiting embodiments of a method of forming a composition will now be disclosed. The method of forming a composition comprise the steps of:

(a) heating a first surfactant, a second surfactant, a third surfactant, cholesterol, an active agent and a first solvent to form a mixture; and

(b) heating the mixture of step (a) with a second solvent and cooling the mixture to form the composition,

wherein the first surfactant has a transition temperature of at least 50 °C, wherein the second surfactant has a transition temperature of less than 20 °C, and wherein the third surfactant has a hydrophilic-lipophilic balance (HLB) value of at least 15.

[0058] In step (a), the first surfactant may be sorbitan monostearate.

5 [0059] The first surfactant may have a weight percentage in the range of about 5.7 weight% to about 74.8 weight%, about 20 weight% to about 74.8 weight%, about 40 weight% to about 74.8 weight%, about 60 weight% to about 74.8 weight%, about 5.7 weight% to about 60 weight%, about 5.7 weight% to about 40 weight%, about 5.7 weight% to about 20 weight% or about 20 weight% to about 30 weight% based on the total weight of the mixture.

10 [0060] The transition temperature of the first surfactant is at least about 50 °C, at least about 55 °C, at least about 60 °C, at least about 65 °C, at least about 70 °C, about 50 °C to about 70 °C, about 50 °C to about 65 °C, about 50 °C to about 60 °C, about 50 °C to about 55 °C, about 55 °C to about 70 °C, about 60 °C to about 70 °C, about 65 °C to about 70 °C.

[0061] In step (a), the second surfactant may be sorbitan monooleate, sorbitan mololaurate or
15 combinations thereof.

[0062] The second surfactant may have a weight percentage in the range of about 2.8 weight% to about 72 weight%, about 20 weight% to about 72 weight%, about 40 weight% to about 72 weight%, about 60 weight% to about 72 weight%, about 2.8 weight% to about 60 weight%, about 2.8 weight% to about 40 weight%, about 2.8 weight% to about 20 weight% or about 10 weight%
20 to about 15 weight% based on the total weight of the mixture.

[0063] The transition temperature of the second surfactant is less than about 20 °C, less than about 15 °C, less than about 10 °C, less than about 5 °C, less than about 0 °C, less than about -5 °C, less than about -10 °C, less than about -15 °C, less than about -20 °C, about -20 °C to about 20 °C, about -20 °C to about 15 °C, about -20 °C to about 10 °C, about -20 °C to about 5 °C, about -
25 20 °C to about 0 °C, about -20 °C to about -5 °C, about -20 °C to about -10 °C, about -20 °C to about -15 °C, about -15 °C to about 20 °C, about -15 °C to about 15 °C, about -15 °C to about 10 °C, about -15 °C to about 5 °C, about -15 °C to about 0 °C, about -15 °C to about -5 °C, about -15 °C to about -10 °C, about -10 °C to about 20 °C, about -10 °C to about 15 °C, about -10 °C to about 10 °C, about -10 °C to about 5 °C, about -10 °C to about 0 °C, about -10 °C to about -5 °C, about
30 -5 °C to about 20 °C, about -5 °C to about 15 °C, about -5 °C to about 10 °C, about -5 °C to about 5 °C, about -5 °C to about 0 °C, about 5 °C to about 20 °C, about 5 °C to about 15 °C, about 5 °C to about 10 °C, about 10 °C to about 20 °C, about 10 °C to about 15 °C, about 15 °C to about 20 °C.

[0064] In step (a), the third surfactant may be polyoxyethylene (20) sorbitan monolaurate.

[0065] The third surfactant may have a weight percentage in the range of about 0.71 weight% to about 69.8 weight%, about 20 weight% to about 69.8 weight%, about 40 weight% to about 69.8 weight%, about 60 weight% to about 69.8 weight%, about 0.71 weight% to about 60 weight%, about 0.71 weight% to about 40 weight%, about 0.71 weight% to about 20 weight% or about 3 weight% to about 4 weight% based on the total weight of the mixture.

[0066] The hydrophilic-lipophilic balance (HLB) value of the third surfactant may be at least about 15, at least about 16, at least about 17, at least about 18, at least about 19, at least about 20, at least about 15 to about 20, at least about 16 to about 20, at least about 17 to about 20, at least about 18 to about 20, at least about 19 to about 20, at least about 15 to about 19, at least about 16 to about 19, at least about 17 to about 19, at least about 18 to about 19, at least about 15 to about 18, at least about 16 to about 18, at least about 17 to about 18, at least about 15 to about 17, at least about 16 to about 17, at least about 15 to about 16.

[0067] In step (a), cholesterol may have a weight percentage in the range of about 0.74 weight% to about 69.8 weight%, about 20 weight% to about 69.8 weight%, about 40 weight% to about 69.8 weight%, about 60 weight% to about 69.8 weight%, about 0.74 weight% to about 60 weight%, about 0.74 weight% to about 40 weight%, about 0.74 weight% to about 20 weight% or about 3 weight% to about 4 weight% based on the total weight of the mixture.

[0068] In step (a), the combination of the first surfactant, the second surfactant, the third surfactant and cholesterol may have a combined hydrophilic-lipophilic balance value in the range of about 5 to 6, about 5 to 5.5 or about 5.5 to 6. In step (a), the active agent may be a hydrophobic molecule.

[0069] The active agent may additionally or alternatively be a drug molecule. The active agent may be an anti-inflammatory agent, an anticancer agent or an anti-infection agent. The active agent may be berberine, capsaicin or a salt or a combination thereof.

[0070] The active agent may have a weight percentage in the range of about 0.9 weight% to about 70 weight%, about 20 weight% to about 70 weight%, about 40 weight% to about 70 weight%, about 60 weight% to about 70 weight%, about 0.9 weight% to about 60 weight%, about 0.9 weight% to about 40 weight%, about 0.9 weight% to about 20 weight% or about 0.5 weight% to about 1 weight% based on the total weight of the mixture.

[0071] In the step (a), the first solvent may be selected from the group consisting of methanol, ethanol, propanol, isopropanol, butanol, 1,2-propanediol, 1,3-propanediol or combinations thereof.

[0072] The first solvent may be isopropanol. The first solvent may be 1,2-propanediol.

- [0073] The first solvent may have a weight percentage in the range of about 10 weight% to about 79.1 weight%, about 20 weight% to about 79.1 weight%, about 40 weight% to about 79.1 weight%, about 60 weight% to about 79.1 weight%, about 10 weight% to about 60 weight%, about 10 weight% to about 40 weight%, about 10 weight% to about 20 weight% or about 15 weight% to about 20 weight% based on the total weight of the mixture.
- [0074] In step (a), the heating may be undertaken at a temperature in the range of about 50 °C to about 80 °C, about 60 °C to about 80 °C, about 70 °C to about 80 °C, about 50 °C to about 70 °C, about 50 °C to about 60 °C or about 60 °C to about 70 °C.
- [0075] The heating may be undertaken for a duration in the range of about 2 minutes to about 8 minutes, about 4 minutes to about 8 minutes, about 6 minutes to about 8 minutes, about 2 minutes to about 6 minutes, about 2 minutes to about 4 minutes or about 4 minutes to about 6 minutes.
- [0076] In step (b), the second solvent may be selected from the group consisting of water, a phosphate buffered saline or combinations thereof.
- [0077] The second solvent may be a phosphate buffered saline.
- [0078] The second solvent may have a weight percentage in the range of about 10 weight% to about 79.1 weight%, about 20 weight% to about 79.1 weight%, about 40 weight% to about 79.1 weight%, about 60 weight% to about 79.1 weight%, about 10 weight% to about 60 weight%, about 10 weight% to about 40 weight%, about 10 weight% to about 20 weight% or about 20 weight% to about 30 weight% based on the total weight of the mixture.
- [0079] In step (b), the heating may be undertaken at a temperature in the range of about 50 °C to about 80 °C, about 60 °C to about 80 °C, about 70 °C to about 80 °C, about 50 °C to about 70 °C, about 50 °C to about 60 °C or about 60 °C to about 70 °C.
- [0080] In step (b), the heating may be undertaken for a duration in the range of about 2 minutes to about 8 minutes, about 4 minutes to about 8 minutes, about 6 minutes to about 8 minutes, about 2 minutes to about 6 minutes, about 2 minutes to about 4 minutes or about 4 minutes to about 6 minutes.
- [0081] In step (b), the cooling of the mixture may be undertaken at room temperature or until the composition reaches room temperature, where room temperature may be about 20°C to about 25°C.
- [0082] Exemplary, non-limiting embodiments of a kit will now be disclosed. The kit may comprise the composition as described herein and instructions for using the composition.
- [0083] The kit may be for use in therapy or in treating a disease in a patient.

- [0084] The disease may be selected from the group consisting of inflammation, cancer, infection or combinations thereof. The disease may be inflammation. The inflammation disease may be osteoarthritis.
- [0085] The composition may be administered by dermal or topical administration.
- 5 [0086] The composition may comprise about 200 mg to about 400 mg of the active agent.
- [0087] The composition may be administered for about 2 to 3 times daily.
- [0088] There is also provided use of the composition as described herein in the manufacture of a kit for the treatment of a disease selected from the group consisting of inflammation, cancer, infection or combinations thereof. The kit may include instructions for using the composition.
- 10 [0089] Exemplary, non-limiting embodiments of a method of treating a disease in a patient will now be disclosed. The method of treating a disease in a patient comprises administering to the patient an effective amount of the composition as described herein, wherein the disease is selected from the group consisting of inflammation, cancer, infection or combinations thereof.
- [0090] The disease may be inflammation. The inflammation disease may be osteoarthritis.
- 15 [0091] The administering step may be undertaken by dermal or topical administration.
- [0092] The composition may comprise about 200 mg to about 400 mg of the active agent.
- [0093] The composition may be administered for about 2 to 3 times daily.
- [0094] Exemplary, non-limiting embodiments of a method of delivering an active agent to a target site will now be disclosed. The method of delivering an active agent to a target site comprises
- 20 the steps of:
- (a) providing a composition comprising the active agent as described herein or as formed according to the method as described herein; and
 - (b) administering the composition at a first site to allow the active agent to move to or be transported to the target site.
- 25 [0095] The active agent may be a hydrophobic molecule. The active agent may additionally or alternatively be a drug molecule. The active agent may be an anti-inflammatory agent, an anticancer agent or an anti-infection agent. The active agent may be berberine, capsaicin or a salt or a combination thereof.
- [0096] The administering step (b) may be undertaken by dermal or topical administration.
- 30 [0097] Where the composition is administered to a dermal site or topically, the composition may be absorbed into the body via the skin and the composition or the active agent may move to or be transported to the target site (thus being delivered to the target site), the target site being the

site where the disease happens or where a symptom of the disease is to be treated. The target site can be a diseased organ or tissue.

[0098] The composition may comprise about 200 mg to about 400 mg of the active agent.

[0099] The composition may be administered for about 2 to 3 times daily.

5 [00100] Exemplary, non-limiting embodiments of a composition for use in therapy will now be disclosed. The present disclosure relates to the composition as described herein for use in therapy.

[00101] The present disclosure relates to the composition as described herein for use in the treatment or prevention of a disease selected from the group consisting of inflammation, cancer, infection or combinations thereof.

10 [00102] The disease may be inflammation. The inflammation disease may be osteoarthritis.

[00103] Exemplary, non-limiting embodiments of use of a composition will now be disclosed. The present disclosure relates to the use of the composition as described herein in the manufacture of a medicament for the treatment or prevention of a disease selected from the group consisting of inflammation, cancer, infection or combinations thereof.

15 [00104] The disease may be inflammation. The inflammation disease may be osteoarthritis.

[00105] The composition may comprise about 200 mg to about 400 mg of the active agent.

[00106] The medicament may be administered for about 2 to 3 times daily.

[00107] The composition may be in the form of a gel, cream, foam, lotion or ointment.

20 [00108] In the present disclosure, a composition (which can take the form of a proniosome gel formulation) is formulated and key parameters including mechanical strength, hydration properties, and drug delivery of the composition are evaluated. The addition of the second surfactant successfully reduced the mechanical strength of the composition (when in the form of the proniosome gel) and improved spreadability on the skin. *In-silico* simulations supported and rationalized these findings. By using a combination of first surfactant, second surfactant and third
25 surfactant, an easily spreadable and readily hydrated composition (such as a gel) was achieved that could efficiently load the active agent for subsequent localized release upon application on the skin, as testified by an *ex-vivo* skin permeation study. By using a combination of first surfactant and second surfactant, a composition (such as a gel) was achieved which has a low critical strain and mechanical strength, resulting in increased spreadability, maximized release of the active
30 agent from the gel and promoted the dermal delivery of the active agent to obtain an optimal pharmacological activity. Furthermore, it was demonstrated that the optimized composition as described herein loaded with the active agent was effective in suppressing pain, attenuating inflammation, and reducing cartilage degradation in *in vitro* and *in vivo* models of OA. These

findings supported the development of gel formulation for delivery of natural compounds as alternatives to NSAIDs for topical treatment of chronic inflammatory and degenerative conditions such as OA.

5 BRIEF DESCRIPTION OF THE DRAWINGS

[00109] The accompanying drawings illustrate a disclosed embodiment and serves to explain the principles of the disclosed embodiment. It is to be understood, however, that the drawings are designed for purposes of illustration only, and not as a definition of the limits of the invention.

10 [00110] **Fig. 1** shows a schematic diagram of a known mechanism of forming niosomes from proniosome gel.

[00111] **Fig. 2** shows a table containing photographs depicting the optimisation of the mass of sorbitan oleate (S80) required to reduce the mechanical strength of sorbitan stearate (S60)-based proniosome gel. Proniosome gel was heated under water bath for 1 minute and the flow of the gel formulation were observed after heating the samples at 1 minute.

15 [00112] **Fig. 3A-3E** shows images and graph depicting physical characterizations of proniosome gel. **Fig. 3A** shows digital images of proniosome gel in upright and inverted positions. **Fig. 3B** shows a SEM image of proniosome gel. The white arrow indicates the niosomes in the formulation. Scale bar: 10 μ m. **Fig. 3C** shows hydration of the proniosome gel and **Fig. 3D** shows a digital image of niosomes under optical microscope. Scale bar: 10 μ m. **Fig. 3E** shows a graphical XRD analysis of the proniosome gel formulations (A – thick black line), (B – medium black line) and
20 (C – thin black line).

[00113] **Fig. 4A-4B** shows a 3D molecular simulation model of the bilayers in the proniosome gel. **Fig. 4A** shows a molecular simulation model of the bilayers in various proniosome gel formulation. **Fig. 4B** shows a schematic diagram illustrating the atomic density map that were
25 derived from the simulation models. The schematic diagram illustrates the mechanism of S80 and T20 in disrupting the close packing of S60 in the bilayers.

[00114] **Fig. 5** shows atomic density maps determined for bilayers composed of only S60 (Formulation (A)). S60 mixed with S80 (Formulation (B)) and S60, S80 and polyethylene glycol sorbitan monolaurate (T20) (Formulation (C)). In all cases some number (2048) of water
30 molecules was added into the simulation model.

[00115] **Fig. 6A-6D** shows graphical rheological characterizations of proniosome gel. **Fig. 6A** shows a chart of G' values (Pa) of proniosome gel Formulations (A), (B) and (C) at 25 °C. **Fig. 6B** shows a graph of shear thinning behaviour of the proniosome gel measured at 32 °C. **Fig. 6C**

shows a graph of dynamic step strain measurement of Formulation (C) where the samples were subjected to 100 % shear strain while increasing the temperature to 32 °C. **Fig. 6D** shows a chart of G' values of Formulations (A)-(C) after subjected to 100% shear strain at 400 s. **P < 0.01, ****P < 0.0001.

5 [00116] **Fig. 7A-7E** shows graphical rheological characterizations of proniosome gel loaded with berberine. **Fig. 7A** shows a graph of amplitude sweep measurements for all three proniosome gel formulations. **Fig. 7B** and **Fig. 7C** show graphs of dynamic step strain measurements of proniosome gels (A) and (B), respectively. **Fig. 7D** shows a graph of an amplitude sweep measurement for Formulation (C) loaded with berberine (Formulation (C) w/BB) in comparison to Formulation (C). **Fig. 7E** shows a graph of a dynamic step strain measurement of berberine loaded in formulation (C) in comparison to Formulation (C).

[00117] **Fig. 8A-8D** shows chart depicting the *ex vivo* skin permeation data of berberine released from various proniosome gels against in-vitro assays on the anti-inflammatory profile and cytotoxicity of berberine. **Fig. 8A** shows a chart depicting the amount of berberine accumulated in the porcine skin after various proniosome gel formulations were applied for 24 hours. **Fig. 8B** shows cytotoxic profile of berberine on HaCaT keratinocytes. **Fig. 8C** and **Fig. 8D** shows charts depicting fold change in nitric oxide and sGAG synthesis observed respectively when inflamed chondrocytes induced by IL-1 β and TNF- α were treated with berberine. The data obtained were normalized against normal chondrocytes (NC). **P < 0.01, ***P < 0.001, ****P < 0.0001 and ns denotes statistically not significant.

[00118] **Fig. 9A-9D** show images and graphs of characterization data of berberine when added into proniosome gel. **Fig. 9A(I)-9A(III)** are digital images of the proniosome gel with or without berberine. **Fig. 9A(I)** is a digital image of Formulation (C), **Fig. 9A(II)** is a digital image of Formulation (C) loaded with berberine and **Fig. 9A(III)** is a digital image of Formulation (C) loaded with berberine in an inverted position. **Fig. 9B** shows a graph plotting the effect of increasing loading of berberine in Formulation (C) of proniosome gel. **Fig. 9C(I)-9C(III)** shows images of niosomes in **Fig. 9C(I)** bright field, **Fig. 9C(II)** fluorescent and **Fig. 9C(III)** overlay. **Fig. 9D(I)-9D(III)** shows images of niosomes in **Fig. 9D(I)**-bright field, **Fig. 9D(II)**-fluorescent and **Fig. 9D(III)** overlay when loaded with berberine. Scale bar of images is at 100 μ m. Microscope images of niosome were taken at 20x magnification while the enlarged image of niosomes in **Fig. 9C(I)** was taken at 50x.

[00119] **Fig. 10A-10B** shows graphs plots of the release profile of berberine from various proniosome gel formulations with the molecular simulation data. **Fig. 10A** shows a chart plotting

the cumulative amount of berberine released from the proniosome gel formulations. **Fig. 10B** shows a graph plotting interaction energy between two bilayers that represent the interaction between niosomes at varying amount of water molecules. The interaction energy was calculated based on molecular simulations.

- 5 [00120] **Fig. 11A-11C** shows charts and graph plotting the application of proniosome gels in an *in vivo* mouse model of OA. **Fig. 11A** shows a graph plotting the time-course analysis of pain behavioral response following the treatment with various proniosome gels. $n = 4$ mice per group. **Fig. 11B** shows a chart plotting the Mankin scores for histopathological assessment of the articular cartilage from mice in various experimental groups at day 6. $n = 4$ mice for Formulations (A)-(C) while $n = 3$ mice for untreated and placebo groups. **Fig. 11C** shows a chart plotting the relative gene expression of pro-inflammatory cytokines detected in the mice joint capsule after mice treated with formulation (C) at day 10 and in comparison, to day 6. Results were normalized against healthy mice joint capsule sample and were expressed as fold change. $n = 4$ and data represented as mean $\pm$ SD. $*P < 0.05$, $***P < 0.001$, and ns denotes statistically not significant.
- 10 **Fig. 11D(I)-11D(VI)** shows images of Safranin-O (Saf-O) staining results of various experimental groups at day 6 after treatment with **Fig. 11D(I)** Formulation (A), **Fig. 11D(II)** Formulation (B), **Fig. 11D(III)** Formulation (C) in comparison to **Fig. 11D(IV)** untreated, **Fig. 11D(V)** placebo-treated and **Fig. 11D(VI)** healthy control. Scale bar: 200 μ m for 40x magnification and 100 μ m for 100x magnification.
- 20 [00121] **Fig. 12A-12B** shows charts of the percentage weight bearing ability of mice in various experimental groups at **Fig. 12A** day 6 and **Fig. 12B** day 10. $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$ and ns denotes statistically not significant.
- [00122] **Fig. 13** shows digital images of formulas A to C when made into a gel according to the method of the disclosure.
- 25 [00123] **Fig. 14** shows digital images of formulas S1 to S3 when made into a gel according to the method of the disclosure.
- [00124] **Fig. 15** shows digital images of various combinations of non-ionic surfactants when made into a gel according to the method of the disclosure. The combinations were heated at various temperatures for 1 minute before the images were captured.
- 30 [00125] **Fig. 16** shows a ternary diagram of the suitable range of components of the proniosome gel composition.
- [00126] **Fig. 17A-17B** shows charts and western blots depicting the effects of berberine on matrix biosynthetic activity of mouse chondrocytes in the presence of IL-1 β and TNF- α . Western

blot analysis showed that berberine restored the synthesis of type II collagen (Col II) (Fig. 17A) and suppressed the production of MMP-13 in IL-1 β /TNF- α stimulated chondrocytes (Fig. 17B). Image LabTM software was utilized to quantify the protein bands and the data were normalized against GAPDH.

5

EXAMPLES

Non-limiting examples of the invention and a comparative example will be further described in greater detail by reference to specific examples, which should not be construed as in any way limiting the scope of the invention.

10 Example 1: Materials

[00127] Berberine Chloride, sorbitan oleate (S80), sorbitan stearate (S60), polyethylene glycol sorbitan monolaurate (T20), buspirone HCl, monosodium iodoacetate (MIA) were purchased from Sigma-Aldrich, Singapore. Isopropyl alcohol (IPA), acetonitrile, dimethyl sulfoxide (DMSO) and methanol were purchased from Fisher UK. (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (MTT) powder and Cholesterol were purchased from Alfa Aesar, 15 UK. Phosphate-buffered saline (PBS) was purchased from Vivantis, US. Water used in these studies was purified using Millipore Direct-Q (Molsheim, France). Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 (DMEM/F12), Dulbecco's Modified Eagle Medium (DMEM), Foetal Bovine serum (FBS) were purchase from Hyclone (Thermo Scientific Inc., Logan, UT, 20 USA) Collagenase II was purchased from Worthington (Lakewood, NJ, USA). ascorbic acid 2-phosphate were purchased from Sigma (St. Louis, MO, USA), and Penicillin-Streptomycin (PS) was purchase from Life Technologies (Carlsbad, CA, USA). TrypLE recombinant cell-dissociation enzymes was purchased from Life Technologies. 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) was purchased from Alfa Aesar.

25 Example 2: Preparation of A Proniosome Gel and Loading with Berberine

[00128] In demonstrating the enhanced release of niosomes from a proniosome gel, sorbitan stearate (S60) was used as a starting material for the proniosome gel. S60 has a transition temperature of 53 °C and this maintains the formulation in a highly ordered gel state at ambient conditions. However, when the proniosome gel is applied onto skin with the conventional skin 30 surface temperature of 32 °C, the gel might have a stiffness that affects its spreadability. It was postulated that the addition of sorbitan oleate (S80), with a transition temperature of 12 °C, could reduce the mechanical strength of S60-based proniosome gel due to its liquid state at ambient temperature. Given that S60 and S80 have an hydrophilic-lipophilic balance (HLB) value of 4.7

and 4.3, respectively, the proniosome gel would be more lipophilic in nature. Hence, to enhance the ability of the gel to hydrate in an aqueous solution, polyethylene glycol sorbitan monolaurate (T20), with a high HLB value of 16.729 was included into the formulation. A proniosome gel was prepared by the following procedures. Briefly, non-ionic surfactants (see Table 1) and cholesterol were added to a glass vial. 125 μ L of IPA was added for Formulations A to C. The mixture was heated at 65 °C for 5 minutes. 80 μ L of PBS was added into the mixture and it was heated at 65 °C for 5 minutes. The samples were taken out and the proniosome gel was left to cool at room temperature and this allowed the formation of the proniosome gel.

[00129] The mass of the proniosome gel was weighed and recorded. To prepare berberine loaded proniosome gel, berberine was added together with the surfactants and IPA mixture and the preparation steps were as described in the previous paragraph.

Example 3: Optimization of Proniosome Gel

Table 1. Components of the proniosome gel formulation at each optimization step.

Formulation	Mass of S60 (mg)	Mass of S80 (mg)	Mass of T20 (mg)	Mass of Cholesterol (mg)	Net HLB value
A	80	-	-	10	4.7
B	80	40	-	10	4.5
C	80	40	11	10	5.5

[00130] The mechanical strength of the proniosome gel was optimized by a stepwise increment of the mass of S80 in the formulation as shown in Fig. 2. The amount of S80 required in the formulation was determined by the ability of the gel to flow towards the bottom of the microtube at a temperature below 35 °C. This indicated that the mechanical strength of the gel was sufficiently reduced when exposed to a higher temperature close to the skin surface temperature of about 32 - 35 °C. The HLB value of the proniosome gel can be estimated based on the percentage mass ratio of the surfactants that contribute to the equivalent percentage of HLB value in the proniosome gel. The HLB value of the proniosome gel was optimized at a value of 5.5. The formula shows the calculation of the HLB value of the gel: $HLB = \sum HLB_i \times f_i$, where the f_i is the mass ratio of the individual surfactant i in the proniosome gel and HLB_i is the HLB value of the surfactant i . 11 mg of T20 was required to achieve an HLB value of 5.5 in the proniosome gel. The amount of T20 was not increased further as higher amounts of cholesterol would be required to form niosomes with T20, and this may reduce the amount of berberine encapsulated in niosomes.

Example 4: Physicochemical Characterization of Proniosome Gel*Scanning Electron Microscopy (SEM) and X-ray Diffraction (XRD) analysis*

[00131] SEM: Samples were mounted on an aluminum sample studs using double-sided copper tapes and Au sputter at 20 mA for 120 s before examination using a Field Emission Scanning
 5 Electron Microscope (JEOL JSM-6700F, Japan) at 10 kV. XRD: Samples were analyzed using an X-ray diffractometer (D8-Advance Bruker, Germany). Samples were scanned from 2° to 40° with a step size of 0.2° and a time per step of 1 s using Cu K α - radiation.

Optical imaging of niosomes in PBS solution

[00132] An amount of 2 mg of proniosome gel was hydrated with 1 mL PBS and the sample
 10 was heated at 45 °C. The aliquot of the sample (10 μ L) was taken out and the niosomes were observed under the Olympus-BX51 microscope (Olympus, Tokyo, Japan).

Rheological studies

[00133] The rheological attributes of the gel were characterized with an Anton Paar Modular Compact Rheometer (MCR) 102 (Temasek Polytechnic, Singapore), using a cone-plate geometry
 15 with a 50 mm diameter and a 2° angle. Three evaluations on the proniosome gel were made at a frequency of 10 rad/s: (1) Amplitude sweep test was conducted over 0.05 – 2 %. (2) Flow sweep was conducted between 0.1 – 100 s⁻¹. (3) Dynamic step-strain test was also conducted at the following strains according to the sequence: (i) 0.03 % at 25 °C (ii) 100 % with temperature increasing from 25 – 32 °C (iii) 0.03 % at 32 °C.

Example 5: In-Silico Modelling of Non-Ionic Surfactants in Proniosome Gel

Calculations were carried out using LAMMPS molecular dynamics engine. The simulation box consisted of 576 +19 surfactant molecules and either 2048 or 4608 water molecules. The 576 surfactant molecules were divided between S60 and S80 molecules, thus the system contains either pure S60 (SP60 system, Formulation (A)) or mixture of S60 and S80 in a ratio of 2:1 (SP60/SP80
 25 system, Formulation (B)). Additionally, 19 T20 molecules were added to SP60/SP80 system leading finally to the third case, i.e. SP60/SP80/T20 system, Formulation (C). The molecules were arranged in the simulation box using self-designed scripts. Generally, every molecule was added in parallel to the existing one, but alternate molecules were flipped to form a head-to-tail alignment. The force field applied for description of surfactants was the generalized amber force
 30 field, gaff. The partial charges were determined according to RESP (Restrained ElectroStatic Potential) methodology. The TIP3P water model together with shake algorithm were used for the modelling of rigid water molecules. The simulations were carried out in NPT statistical ensemble

using Nose-Hoover barostat. The pressure and temperature were set to 1 bar and 305 K, respectively. The calculations were continued for about 40 ns.

Example 6: Quantification of Berberine Penetration in An Ex Vivo Porcine Skin Model

[00134] Porcine ear skin was used as a model for the skin permeation studies. The skin was harvested, and the subcutaneous fat was removed using a scalpel. The thickness of the skin specimens ranged between 1.38 mm and 1.98 mm. The skin tissues were cut into small portions with an area of 2.5 cm x 2.5 cm and were hydrated with 5 mL of PBS for 30 minutes before the start of experiment. 30 mg of various proniosome gel formulations were loaded onto the donor chamber of the Franz cell and the permeation study was carried out for 24 hours. Thereafter, the porcine skin specimens were removed from the diffusion cell. The stratum corneum of the skin samples were wiped with gauze to remove excess test samples. The skin samples were minced and immersed in 4:1 acetonitrile/methanol to extract berberine from the skin specimen and 50 nM of buspirone HCl was added as an internal standard (IS) for LC-MS/MS analysis.

[00135] LC-MS/MS analyses were carried out using Agilent 1290 Infinity Liquid Chromatography system (Agilent Technologies, Santa Clara, CA, USA), connected to an ABSciex QTRAP R 5500 mass spectrometer (AB Sciex, Framingham, MA, USA), and equipped with a TurbolonSpray source (AB Sciex). Chromatographic separation was carried out using ACQUITY UPLC® BEH C18 column (1.7 µm, 2.1 mm x 50 mm), protected with a guard column (ACQUITY UPLC® BEH C18 VanGuard Pre-column, 1.7 µm, 2.1 mm x 50 mm) (Waters, Milford, MA, USA). The samples prepared were eluted through a column with the mobile phase consisting of ultra-pure water with 5 mM ammonium acetate (solvent A) and acetonitrile/methanol (4:1) (solvent B), with a gradient elution programme of solvents A and B as followed: 90:10 at 0 min; 70:30 at 1 minute; 20:80 at 2 minutes; 90:10 at 5 minutes. For the mass spectrometry (MS), all analyses were performed in electrospray ionization (ESI) positive mode. The source parameters for ESI were as followed: Ion spray voltage: +4000V, source temperature: 650 °C, curtain gas: 20 psi, GS1 (sheath gas): 45 psi, GS2 (drying gas): 55 psi, collision gas (nitrogen) medium. The compound dependent MS parameters were presented in Table 2 below:

Table 2. Compound dependent MS parameters for berberine and buspirone.

Analyte	Q1 Mass (Da)	Q3 Mass (Da)	DP (Volts)	EP (Volts)	CE (Volts)	CXP (Volts)
Berberine	337.300	321.200	99.80	7.24	37.27	7.12
Buspirone (IS)	386.00	122.00	100.00	10.00	37.00	8.00

Example 7: Cell Viability Assay of Keratinocytes

[00136] HaCaT keratinocytes could be purchased from Cell line service (Eppelheim, Germany). Keratinocytes were maintained in DMEM supplemented with 10% FBS under a humidified atmosphere of 5% CO₂ at 37 °C. The medium was changed every alternate day. Upon confluency, keratinocytes were dissociated using trypsin and the cells were harvested for cell viability assay. In the cell viability assay, 2 x 10⁴ HaCaT keratinocytes were seeded with 100 µL of growth medium in each well and the cells were incubated for 24 hours at 37 °C. 100 µL of the growth medium was aspirated and replaced with various concentrations (0.4 - 200 µM) of berberine in the growth medium. The cells were incubated at 37 °C for 48 hours. Subsequently, the growth medium containing berberine were replaced with 100 µL serum free growth medium containing 0.5 % (w/v) of MTT and the cells were incubated for 45 minutes at 37 °C. The medium was removed, and the formazan crystals were dissolved with DMSO for 10 minutes. The absorbance readings measuring the concentration of formazan crystals were taken with Synergy H1 hybrid multimode microplate reader (Bio-Tek, Winoosky, VT, USA) at 570 nm.

Example 8: In Vitro Chondrocyte OA Model

[00137] Primary chondrocytes were harvested from femoral head articular cartilage of 7-week old BALB/c female mice, following the procedure as follows. Briefly, the cartilage tissues were washed 3 times with PBS and digested with 0.2% (w/v) collagenase II overnight at 37 °C. The cells were then passed through a 40-µm cell strainer (Corning®, Corning, NY, USA) to disperse the cells into single cells before seeding at a density of 2 x 10⁴ cells/cm². The culture was maintained in DMEM-F12 supplemented with 10% FBS, 25 µg/ml ascorbic acid 2-phosphate, and 1% PS under a humidified atmosphere of 5% CO₂ at 37 °C. The medium was changed every alternative day. Upon confluency, chondrocytes were dissociated using TrypLE (Life Technologies) and further sub-cultured to passage 2 (P2) cells for in-vitro experiments.

[00138] To assess the anti-inflammatory effects of berberine against OA, an *in vitro* chondrocyte model of OA was employed with modifications. Briefly, P2 mice chondrocytes were seeded in 24-well plate at a density of 2 x 10⁴ cells/cm² and cultured for 16 hours before the medium was changed to a low serum culture medium comprising of DMEM-F12 supplemented with 0.5% FBS and 1% PS for 24 hours. The chondrocytes were stimulated with 5 ng/mL interleukin (IL)-1β and 5 ng/mL tumor necrosis factor (TNF)-α for 1 hour, and then treated with varying concentrations of berberine or vehicle (PBS) for 24 hours. At the end of treatment, cells and culture supernatants were collected for nitric oxide (NO) and sulphated glycosaminoglycan (sGAG) quantification. All *in vitro* experiments were performed in triplicate (n = 3) in at least two independent experiments.

Example 9: Quantification of Sulphated Glycosaminoglycan and Nitric Oxide in An In Vitro OA Model

[00139] Sulphated glycosaminoglycan (sGAG) was measured by digesting the cell pellets ($n = 3$) with proteinase K digestion buffer (100 $\mu\text{g/mL}$ in Tris-HCl buffer, pH 8) for 18 hours at 60 °C. sGAG was measured using Biocolour Blyscan Glycoaminoglycan Assay Kit (Bicolor Ltd, Carrickfergus, UK) as per manufacturer's instruction. The dye in the samples were measured together at 656 nm, together with the standards using a microplate reader (Infinite® 200 PRO, Tecan™, Männedorf, Switzerland). Nitric oxide (NO) was measured in the cell culture supernatants using the Griess reaction assay and nitrite standards from the Griess reagent kit (ThermoFisher Scientific) according to the manufacturer's protocol. The absorbance readings were obtained at 548 nm using a microplate reader (Infinite® 200 PRO). The concentration of sGAG and NO was expressed as fold change by normalizing against the control group that was not stimulated with IL-1 β and TNF- α .

Example 10: Efficacy in A Mouse OA Model

[00140] All procedures were performed according to the Institutional Animal Care and Use Committee at National University of Singapore under the protocol number: R18-1188. A total of forty-eight 6-7 weeks old female BALB/c mice with a mean weight of 18.0 ± 0.5 g were used in this study. The mice were randomly allocated into 6 groups: OA + Formulation (A), OA + Formulation (B), OA + Formulation (C), OA + no treatment, OA + Formulation (C) placebo and healthy mice. OA was induced in the right knee by intra-articular injection of 0.5 mg of MIA dissolved in 10 μL of PBS, as previously described. Three days after inducing OA, the mice received their treatment according to their assigned groups for 7 days. The knee joint was cleaned with saline solution daily before applying 10 mg of proniosome gel onto the skin surface of the knee joint daily for 7 days. The mice were housed under controlled temperature with a 12-hour light and 12-hour dark cycle. They were allowed free movement and access to food and water.

Pain behavioural measurement

[00141] Changes in the hind paw weight distribution between the right OA and left contralateral control limbs were utilized as an index of joint pain in the OA knee. An incapacitance tester (Bioseb, Vitrolles, France) was used for measurement of the hind paw weight distribution. During the static weight bearing test, the mice were placed in a Perspex chamber and the animal was comfortably maintained in a way that their two hind paws were resting on two separate transducer pads. Three 5-second readings of the force exerted by each hind limb of the mouse were recorded.

Results were calculated as the weight borne on the right OA limb as a percentage of total weight borne by the mouse using the equation:

$$\frac{\text{Weight borne on right hind limb}}{\text{Weight borne on right+left hind limb}} \times 100\%$$

A value of 50% represents an equal weight distribution across both limbs and a value of less than 50% indicates that there is a reduction in weight borne on right hindlimb. Baseline measurements were recorded prior to the injection of MIA and the mice were assessed daily from day 3 to day 10 after injection.

Quantitative reverse transcription polymerase chain reaction

Total RNA was isolated from the joint capsule on the mouse knee joint with the RNeasy Mini Kit (Qiagen, Hilden, Germany) following manufacturer's instruction. cDNA was synthesized with 100 ng total mRNA using QuantiTect-Reverse Transcription Kit (Qiagen). qPCR was performed in triplicate with iTaq Universal SYBR Green Supermix and measured in QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Relative mRNA expression levels were normalized against glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNA, calculated using the comparative Δ CT method and were expressed as fold change against the mice without OA.

Histological analysis and scoring of the mouse knee joint

At day 6 of the experiment, animals were euthanized, and the right knee joint were removed surgically, followed by fixation in 10% formalin fixation for 1 week and decalcification in 30% (v/v) formic acid for 2 weeks before proceeding to histology. The samples were dehydrated and embedded in paraffin for microtomy according to the standard procedure. Serial sections were cut at 5- μ m and stained with Safranin-O/Fast green (Saf-O) for sGAG deposition. The stained sections were then imaged using an inverted microscope (Olympus IX70, Olympus, Tokyo, Japan). Based on Saf-O-stained sections, the quality of joint repair was assessed for parameters including cartilage structure, cellularity, matrix staining and tidemark integrity using the Mankin's histological scoring system. The score for healthy mouse cartilage is 0 and the maximum score for degenerative articular cartilage is 14.

Example 11: Statistical Analysis

All statistics were performed using GraphPad Prism 9 software (GraphPad Software Inc., La Jolla, CA, USA). The datasets were checked for normality by using Shapiro–Wilk test. All data were represented as mean $\pm$ standard deviation. Ordinary one-way or two-way ANOVA followed by Turkey *post hoc* test was used to assess the statistical differences between the three or more groups for normally distributed data. For non-normally distributed data, statistical

differences between three or more groups were analyzed using Kruskal-Wallis test, followed by Dunn-Bonferroni *post hoc* test. Spearman's correlation coefficient was used to evaluate the association between two variables, and the data presented as r (Spearman correlation coefficient), n (sample size) and P (statistical significance level). Statistical significance was set at $P < 0.05$.

5 **Example 12: Physical Appearance and Morphological Characterization**

[00147] Over the past decade, proniosomes were reported to be a suitable platform for dermal delivery of bioactive molecules. The presence of non-ionic surfactants enhanced the permeability of compounds through the skin and this increased their accumulation locally. This is expected to preserve the therapeutic efficacy of the compound and reduce the potential of side effects arising from systemic administration. The optimization of the proniosome gel were described in Example 3 and the composition of the formulation summarized in Table 1.

The images of the proniosome gel prepared by using the coacervate phase separation method are presented in Fig. 3A. The proniosome gel appeared as a white layer and the gel structure remained intact when the glass vial was inverted (Fig. 3A). The morphology of proniosome gel was later examined using SEM and a number of densely packed niosomes of vesicular shape were observed in the formulation (Fig. 3B). This confirmed that the non-ionic surfactants assembled into vesicular niosomes before forming the proniosome gel formulation. The proniosome gel was then hydrated with PBS (Fig. 3C) and niosomes were successfully released from the proniosome gel when the solution was observed under the optical microscope (Fig. 3D). The effect of different types of non-ionic surfactants on the crystallinity of the proniosome gel formulation was further assessed using XRD. In Fig. 3E, the XRD pattern of Formulation (A) showed a Bragg peak 2θ angle of 22 (black line), which may represent some extent of liquid crystallinity. The distinct Bragg peak can be said to be caused by the long linear alkyl chain of S60, which contributes to the highly ordered packing structure of the proniosome gel. When S80 was added into Formulation (B), considerable changes were observed in the XRD pattern, with the broadening of a second Bragg peak at a 2θ angle of 19 (medium black line), while diminishes the peak at a 2θ angle of 22 decreased. The addition of T20 in Formulation (C) further diminished the Bragg peaks (green line) at a 2θ angle of 22. Overall, there was a distortion of the original macromolecular arrangement of S60 when both S80 and T20 were added into the formulation. This could be attributed to the more linear structure of S60 as compared to S80 and T20. The inclusion of these two surfactants into the gel may have affected packing of S60 molecules. To substantiate this reasoning, molecular dynamic simulations were performed to study the molecular interactions of the non-ionic surfactants in the proniosome gel, as presented in Example 13.

Example 13: Molecular Dynamic Simulation of The Non-Ionic Surfactants

Molecular packing of non-ionic surfactants

Simulations of the bilayers in the proniosome gel, representing Formulations (A)-(C) were carried out using slab geometries with an approximate volume of $75 \times 75 \times 75 \text{ \AA}^3$ as shown in Fig. 4A.

- 5 From the simulation, the atomic density maps of the considered systems were retrieved (Fig. 5). This quantity was determined as a mean number of atoms found at a given position ($x+dx, y+dy$) of the cross sectional area of a bilayer as illustrated in Fig. 4B. The atomic density map does not distinguish which molecule a given atom belongs to and it can be useful in the analysis of the atomic structure of the formed bilayers. Looking at the obtained patterns of the atomic density
- 10 maps for the three cases shown in Fig. 5, it was noticed that any modification of the initial Formulation (A) (pure S60) led to stronger and stronger disruption of the atomic structure of the bilayer. In the case of Formulation (A), very sharp and densely packed spots could be seen corresponding to standing vertically hydrophobic parts of the surfactants (see Fig. 4A). The spots were very sharp and form a hexagonal-like lattice. This meant that the bilayer formed by pure S60
- 15 in Formulation (A) was very stiff and dense and the distances between individual S60 molecules did not change in time. Addition of S80 into S60 in Formulation (B) led to significant deterioration of the bilayers contributed by S60, which was illustrated by a more diffuse pattern of density map. In this case, the spots were wider and there were areas of reduced density located mainly around S80 molecules. This meant that the structure of the bilayer was more loosely packed and probably
- 20 more permeable towards small molecules. The distortion of the regular S60 bilayer structure was due to the *cis*-alkene functional group of S80 on its alkyl chain. This mechanism was illustrated as a schematic diagram shown in Fig. 4C. The so-obtained distortion and the enhanced permeation of the bilayer were expected to improve the gel spreadability on the skin, and to reduce the mechanical strength of the proniosome gel, as confirmed by the rheology studies in Example 14.
- 25 The reduction of the mechanical strength was also confirmed by values of mean pair interaction energy between atoms within bilayer obtained from the simulations. These values were $-2.56 \text{ kJ mol}^{-1}$, $-2.38 \text{ kJ mol}^{-1}$, and $-2.30 \text{ kJ mol}^{-1}$ for Formulations (A), (B) and (C) respectively. The changes in energy (calculated per single atom) indicated that attraction between a given pair of atoms was weaker when S60 bilayer was modified by addition of either S80 or both S80 and T20.

30 *Wetting property of the bilayers in relation to hydration*

[00148] T20 was added into the proniosome gel to improve the ability of the gel to hydrate itself and to enhance the release of niosomes from the gel formulation. Molecular simulations were

performed to evaluate the ability of T20 to absorb water into the bilayers of niosomes in the proniosome gel. Table 3 presents the calculations from the simulation results.

Table 3. Mean interaction energy of water with the bilayers calculated per single water molecule. A total of 4068 water molecules was added into the simulation model for the quantification of the mean interaction energy.

Parameters	Formulation (A)	Formulation (B)	Formulation (C)
Solvent accessible surface area (SASA) (nm ²)	246±4	268±4	312±8
Ratio of SASA relative to formulation (A)	1.00	1.09	1.27
Mean interaction energy of water with the bilayers (kJ mol ⁻¹)	8.65±0.06	8.59±0.11	9.69±0.11

[00149] The solvent accessible surface area (SASA) describes the area at the interface of the bilayers that are available to interact with water molecules. When S80 and T20 were added into the bilayer simulation model of Formulation (A), the SASA increased from $246 \pm 4 \text{ nm}^2$ to $268 \pm 4 \text{ nm}^2$ (Formulation (B)) and $312 \pm 8 \text{ nm}^2$ (Formulation (C)), respectively. This might suggest that the surface of the bilayer became more corrugated, and this supported the observation from the atomic density map as shown in Fig. 5. The corrugated surface increased the SASA at the interface of the bilayers and allowed the bilayers to adsorb more water. This phenomenon was expected to increase the ability of the proniosome gel to hydrate itself and release the niosomes from the gel formulation. As presented in Table 3, the interaction energy between water and the bilayers representing Formulations (A) and (B) were almost identical ($8.65 \pm 0.06 \text{ kJ mol}^{-1}$ and $8.59 \pm 0.11 \text{ kJ mol}^{-1}$, respectively). This meant that the nature of the external part of the bilayer surface did not change significantly in terms of interactions with water upon the addition of S80. This was expected since the hydrophilic portion of both S60 and S80 had a similar chemical structure. In the case of Formulation (C), the interaction energy was the highest ($9.69 \pm 0.11 \text{ kJ mol}^{-1}$) among the three formulations and this can be attributed to the addition of T20 that increased the hydrophilicity of the bilayers and enhanced the adsorption of water molecules in the bilayers. Collectively, these results supported the strategy of adding T20 to improve the hydration ability of the proniosome gel.

Example 14: Rheological Characterization

[00150] The distortion of the macromolecular packing of S60 by S80 and T20 as verified in the molecular simulation of the proniosome gel (Fig. 3A-3E) may reduce the mechanical strength of the gel. Hence, rheological characterization was performed to quantify the mechanical strength of the various formulations and the results are shown in Fig. 6A-6D.

[00151] In the amplitude sweep measurement, as shown in Fig. 7A, it was noticed that all proniosome gel formulations had higher elastic characteristics, with G' value higher than G'' value. This demonstrated that all the proniosome gels, Formulations (A)-(C) predominantly display the semisolid-like behavior, as supported by Fig 3A. The critical strain for Formulation (A) is at 0.15% while Formulations (B) and (C) had a critical strain of 0.30% and beyond this strain, there was disruption of the microstructure of the proniosome gel. A shear strain of 0.03% was thus selected for subsequent rheological characterization as it fell within the linear viscoelastic region. The higher critical strain observed in Formulations (B) and (C) can be attributed to the presence of the *cis*-alkene functional group on the alkyl chain of S80, which reduced the rigidity of the proniosome gel and allowed it to be more malleable under higher shear strain. Indeed, the reduction of the mechanical strength can favor the release of niosomes from the formulation. It was observed that Formulation (A) had a G' value of 168 ± 50.4 kPa while Formulation (B) had a G' value 8.90 ± 0.74 kPa ($P < 0.0001$) and (C) had a G' value of 9.21 ± 3.27 kPa ($P < 0.0001$) as shown in Fig 6A. This showed that the addition of S80 reduces the mechanical strength of the gel by $\approx 95\%$ most likely by disrupting the highly ordered structure of S60 in the proniosome gel. This finding is supported by the XRD data presented in Fig 3E. Of note, the addition of T20 did not significantly alter the mechanical strength of the gel.

[00152] In the context of topical dermal applications, proniosome gel with shear thinning properties was advantageous as it allowed the ease of spreading the formulation onto the skin. As shown in Fig. 6B, there was a reduction of viscosity for all three proniosome gels with an increasing shear rate. Formulation (A) had the highest viscosity while Formulation (C) had the lowest viscosity, and this shows that it is easier to spread Formulation (C) onto the skin. It was expected that Formulation (C) would retain on the skin and provide continuous release of the encapsulated compound on the application area. Thus, it was imperative for the disrupted macromolecular structure of the formulation to recover its viscoelastic properties after the application of a shear force. Using the dynamic step-strain amplitude test to simulate the application of the gel onto the skin, it was observed that the G' value was reduced to about 1 Pa, when Formulation (C) was subjected to 100% shear strain with increasing temperature up to 32

°C (Fig. 6C). It was also observed that $G'' > G'$, which indicated that the formulation behaved predominantly more liquid like during the application of the shear strain, and this confirmed the ease of spreading for Formulation (C). When the shear strain reverted to the original strain, it was observed that the formulation displayed a certain degree of structure recovery: more precisely, the G' value of Formulation (C) immediately increased to about 480 Pa and exceeded G'' value by about 7 folds. The recovery of the mechanical properties of Formulation (C) might indicate its advantage to be retained onto the skin after the application. As a comparison, dynamic step-strain amplitude test was carried out for both Formulations (A) and (B) (Fig. 7B and Fig. 7C). It was observed that the recovery of the G' value after shear strain (Fig. 6D) (Formulation (A): 7.54 ± 2.53 kPa, Formulation (B): 1.54 ± 0.42 kPa ($P < 0.01$), Formulation (C): 0.48 ± 0.29 kPa ($P < 0.01$)), for all three formulations, showed a similar trend as compared to Fig. 6A. This might influence the dermal delivery of the active ingredient upon the *in vivo* application. In subsequent characterizations, Formulation (C) was compared to Formulations (A) and (B) in terms of gel-like consistency, spreadability and ability to hydrate, and it was used for loading of berberine in the subsequent experiments.

Example 15: Correlating Ex Vivo Skin Permeation Studies with In Vitro Assays to Determine The Therapeutic Efficacy and Cytotoxicity of Berberine

[00153] The proniosome gel was expected to be applied onto the skin to achieve a localized delivery of berberine at the OA knee joint. It was also expected that the amount of water on the skin would be much lower and the release profile of berberine from the proniosome gel might differ substantially in actual applications. Hence, the release of berberine from various proniosome gels was measured by setting up a more realistic *ex vivo* skin permeation study using porcine ear skin. Fig. 8A showed the accumulation of berberine on the skin from the various proniosome gel formulations.

[00154] Prior to the skin permeation studies, the amount of berberine loaded in the proniosome gel was optimized as shown in Fig. 9A and Fig. 9B. The addition of berberine did not significantly alter the rheological characteristics of the proniosome gel (Fig. 7D and Fig. 7E) and fluorescent signals of berberine were observed in the niosomes when the formulation was hydrated in PBS (Fig. 9C and Fig. 9D). This confirmed that berberine was confined within the niosome vesicles and not just dispersed within the gel matrix. The percentage encapsulation efficiency of the niosomes were shown in Fig. 9E.

Table 4. Quantification of berberine embedded in proniosome gel Formulations (A)-(C) and the percentage encapsulation efficiency of berberine in niosomes.

Formulation	Amount of Berberine embedded in proniosome gel (mg)	Amount of berberine encapsulated in niosomes (mg)	Percentage encapsulation (%)
(A)	1746 ± 83.7	1167 ± 71.1	66.7 ± 1.3
(B)	1487 ± 76.9	968 ± 70.2	65.2 ± 4.5
(C)	1379 ± 85.9	833 ± 83.2	60.3 ± 2.4

[00155] Although Formulation (C) had the lowest berberine concentration (13.7 µg/mg, Table 4), the formulation had the highest amount of berberine released at 1.02 ± 0.15 µg as compared to Formulations (B) and (A), at 0.65 ± 0.23 µg ($P < 0.01$) and 0.30 ± 0.04 µg ($P < 0.001$), respectively (Fig. 8A). However, the amount of berberine released was substantially lowered when compared to the amount of berberine released from the proniosome gel (~ 150 µg) when hydrated under excess PBS (Fig. 10A). The difference can be attributed to the excess PBS that reduced the interaction energy between the bilayers (Fig. 10B) and allowed berberine to be released more effectively from the formulation. Nonetheless, the amount of berberine released in porcine skin model highlight the need for optimization of the mechanical strength and hydrating ability of the gel to enhance the absorption of the limited amount of water in the skin, to increase the release of niosomes and active molecules from the formulation.

[00156] It was recognised that the overall amount of berberine released from the formulations was extremely low as compared to the original concentration of berberine in the proniosome gel. As such, it was investigated whether the released berberine could still induce any anti-inflammatory effects and reduce cartilage degradation. Concurrently, the cytotoxicity profile of berberine was tested on HaCaT keratinocytes and the results in Fig. 8B showed that the inhibitory concentration of berberine at 50% cell viability (IC_{50}) was approximately 33 µg mL⁻¹. This result is encouraging, as the range of berberine concentrations responsible for cytotoxicity towards skin cells is much higher than the amount required to display a pharmacological effect.

[00157] Indeed, when the anti-inflammatory effect of berberine was tested in an *in-vitro* chondrocyte model of OA, the results (Fig. 8C) showed that at 10 µg mL⁻¹ of berberine had significantly reduced the concentration of NO to 6-folds ($P < 0.0001$) relative to the control group (11-fold change) that were stimulated with IL-1β and TNF-α. Moreover, this concentration did not significantly reduce the cell viability of the chondrocytes (data not shown) and was sufficient to

restore the production of sGAG, to a level comparable to that of healthy chondrocytes ($P > 0.99$) (Fig. 8D).

Example 16: Activity of berberine on OA biomarkers

[00158] Sulphated glycosaminoglycan (sGAG) is a composition of proteoglycan that exists naturally in the extracellular matrix of cartilage tissue. Here, sGAG was used as a chondrocyte marker to evaluate the effect of gel/noisome system for the treatment of osteoarthritis (OA). Besides sGAG, Col II is a major extracellular matrix component of cartilage, which can be degraded by metalloproteinases-13 (MMP13) during OA.

[00159] A western blot analysis was performed to detect the production of type II collagen and matrix metalloproteinase-13 (MMP13) in the cells treated with varying doses of berberine (Fig. 17A-17B). The western blotting results showed type II collagen that was attenuated by IL1- β /TNF- α treatment in the *in vitro* OA chondrocyte model was reversed by treatment with berberine from concentration as low as $1 \mu\text{g mL}^{-1}$, restoring to comparable levels as the normal chondrocytes (Fig. 17A). Additionally, berberine treatment inhibited the production of MMP13 induced by IL1- β /TNF- α treatment, with $10 \mu\text{g mL}^{-1}$ concentration demonstrating the most potent effects on suppressing the production of MMP13 (Fig. 17B).

Example 17: Optimized Berberine-Loaded Proniosome Gel Suppresses Pain, Attenuates Inflammation, and Reduces Cartilage Degradation

[00160] Next, the therapeutic effects of the proniosome gel Formulations (A)-(C) loaded with berberine were investigated in the mouse model of OA. MIA was used to induce OA for 3 days. Thereafter, the OA mice received topical treatment with the various gel formulations. Mice without OA served as healthy control, while untreated or placebo-treated OA mice served as negative controls.

[00161] Nociceptive responses by weight distribution measurements were assessed daily during treatment. In healthy mice without OA, there was an equal weight distribution on both hind limbs, and this corresponded to 50% of the weight on each limb (Fig. 11A). Among the OA mice that received the different proniosome gel formulations, OA mice that received Formulation (C) observed the improvements in weight bearing ability from $31.2 \pm 2.6\%$ at day 3 to $43.1 \pm 0.7\%$ ($P < 0.0001$) as early as day 6 before plateaued thereafter with $43.1 \pm 0.7\%$ at day 10 (Fig. 12A and Fig. 12B). On the other hand, the percentage weight distribution for mice that received Formulation (B) was $36.1 \pm 3.2\%$ and Formulation (A) was $31.4 \pm 2.9\%$ at day 6 (Fig. 12A) and hovering around 40% at day 10 (Fig. 12B). In contrast, the percentage weight distribution of untreated OA or placebo-treated OA mice hovered around 30 – 35% from day 3 to day 10.

[00162] Consistent with the observations that Formulation (C) had the optimal anti-nociceptive effects, histopathology assessment confirmed that OA mice treated with Formulation (C) had minimal cartilage degradation. As evidenced by the Saf-O staining results, OA mice treated with Formulation (C) had higher amounts of sGAG deposited in the cartilage (Fig. 11D(III)) in comparison to OA mice treated with Formulations (A) (Fig. 11D(I)), (B) (Fig. 11D(II)) or left untreated (Fig. 11D(IV)). Consistently, in OA mice treated with Formulation (C) were observed improvements for parameters including cartilage structure, cellularity, matrix staining and tidemark integrity that culminated in a Mankin score of 2.3 ± 1.0 , significantly better than that of Formulation (A) with 4.0 ± 0.8 and Formulation (B) with 3.3 ± 1.0 (Fig. 11(b)). In contrast, the untreated OA and placebo-treated OA mice had Mankin scores of 6.3 ± 2.3 and 4.3 ± 0.6 , respectively. By Spearman's correlation analysis, our results suggest that the improvements to the weight bearing ability on the OA knee ($r = -0.545$, $n = 18$, $P = 0.019$) were inversely correlated with the decrease in Mankin scores ($r = -0.515$, $n = 18$, $P = 0.028$) for OA degenerative changes. The improvements to the weight bearing ability on the OA knee and reduced cartilage erosion can be attributed to the enhanced penetration of berberine through the skin, as earlier supported by skin permeation studies (Fig. 8A).

[00163] Since Formulation (C) loaded with berberine demonstrated the most potent effects in suppressing pain and reducing cartilage degeneration, its effects on OA inflammation were further investigated. Here, the joint capsule on the mouse OA knee was harvested to evaluate the gene expression of pro-inflammatory cytokines including TNF- α , IL-1 β and IL-6 which were key pro-inflammatory cytokines involved in the cartilage destruction in OA. As shown in Fig. 11C, the delivery of berberine in Formulation (C) significantly reduced the gene expression of IL-6 from ~60-fold change to ~5-fold change while gene expression for TNF- α and IL-1 β remained unchanged between day 6 and day 10. This finding suggested that berberine attenuates inflammation in OA by inhibiting the expression of IL-6. This result demonstrated the anti-inflammatory properties of berberine in inhibiting the secretion of IL-6 from lipopolysaccharide (LPS)-stimulated macrophages *in vitro*. Collectively, the results suggested that Formulation (C) had efficiently delivered berberine through the skin and this allowed berberine to exert its anti-inflammatory activity to alleviate pain and reduce cartilage degeneration in OA.

30 **Example 18: Methods of Characterisation and Optimisation**

Reduction of the mechanical strength of S60-based proniosome gel using S80

[00164] The percentage mass ratio of S60 and S80 was optimized by a stepwise increment of the mass of S80 by 10 mg as shown in Fig. 2. The different mixtures were used to formulate the

proniosome gel and the flow behaviour of the proniosome gel was observed at different temperatures by heating the samples in the water bath for 1 minute. The percentage mass ratio of S60 and S80 was selected based on the flow behaviour of proniosome gel below 35 °C.

Quantification of berberine embedded in proniosome gel

- 5 [00165] 100 mg of the proniosome gel was hydrated with 20 mL PBS at 45 °C and the sample was vortexed for 5 minutes to ensure complete dissolution of the gel. An aliquot of the sample (100 µL) was taken and berberine was extracted using 100 µL of the solvent containing ACN: MeOH in 1:1 ratio. The mixture was vortexed, heated at 80 °C, and centrifuged to remove the surfactants. The absorbance of the supernatant was read with a microplate reader (Bio-Tek,
10 Winoosky, VT, USA) at 346 nm.

Quantification of berberine encapsulated in niosomes

- [00166] 100 mg of the proniosome gel was hydrated with 20 mL PBS at 45 °C and the sample was vortex for 5 minutes to ensure a completed conversion of the gel into niosome vesicles. The sample was further dialysed in a dialysis bag, with a molecular weight cut-off (MWCO) at 3.5
15 kDa, at 4 °C to remove the unencapsulated berberine. An aliquot of the sample (100 µL) was taken out at various time points and the absorbance of the sample was read with a microplate reader (Bio-Tek, Winoosky, VT, USA) at 346 nm to analyze the amount of berberine retained in the sample. The encapsulation efficiency of the niosome vesicles was determined upon confirming that the concentration of berberine in the sample remained constant in the dialysis bag.

20 *Optical and fluorescence imaging of berberine in niosomes*

- [00167] An amount of 2 mg of berberine loaded proniosome gel was hydrated with 1 mL PBS and the sample was heated at 45 °C. The aliquot of the sample (10 µL) was taken out and the niosomes were observed under the Olympus-BX51 microscope (Olympus, Tokyo, Japan). Fluorescence images of berberine were taken using the green channel and the images were taken
25 at 20x magnification. The size of the niosomes was measured using ImageJ software (National Institute of Health, Bethesda MD, USA).

Release profile of berberine released from the proniosome gel

- [00168] 120 mg of various proniosome gel formulations loaded with berberine were placed in a holder. Each gel formulation was immersed individually in 15 mL of phosphate buffer saline
30 solution at 32 °C. At each time point, an aliquot of solution (100 µL) was taken out and the sample was centrifuged to remove any remaining gel. The absorbance of the supernatant was read with a microplate reader at 346 nm to determine the concentration of berberine released from the formulation.

Comparative Example 1: Comparisons between Formulations of Different Non-Ionic Surfactants

[00169] Various combinations of non-ionic surfactants were processed according to the method of preparing a proniosome gel as described above. As shown in Fig. 13 to Fig. 15, S80 and its combination with T20 formed viscous liquids instead of gels. S60 and its combination with T20 had a high stiffness when formed into gels, which are not suitable for medical applications.

The formulation was prepared according to the table below:

Table 5. Comparison between proniosome gel formulations having different surfactant combinations.

Formulation	Mass of S60 (mg)	Mass of S80 (mg)	Mass of T20 (mg)	Mass of Cholesterol (mg)
A	80	-	-	10
B	80	40	-	10
C	80	40	11	10
S1	-	80	-	10
S2	-	80	11	10
S3	80	-	11	10

[00170] A proniosome gel was prepared by the following procedures. Briefly, non-ionic surfactants (see Table 5) and cholesterol were added to a glass vial. 125 μ L of IPA was added and the mixture was heated at 65 °C for 5 minutes. 80 μ L of PBS was added into the mixture and it was heated at 65 °C for 5 minutes. The samples were taken out and the proniosome gel was left to cool at room temperature and this allowed the formation of the proniosome gel.

[00171] As shown in Fig.13, all three types of proniosome gel formulation (A to C) had successfully formed the proniosome gel and this demonstrated that the addition of Span® 80 and Tween® 20 did not affect the gelation ability of Span® 60 as the formulation remains as a semi-solid when the sample was inverted. As a control, formulations S1 and S2 were prepared to evaluate the gelation ability of Span® 80. As shown in Fig. 14, both S1 and S2 formulations appeared as a viscous liquid when the sample was inverted, and this demonstrated that a semi-solid formulation was not formed. The viscous liquid formulation may not be a suitable formulation as the active agent in the formulation may prematurely release from the formulation. In formulation S3, Tween® 20 was added to evaluate its ability to reduce the mechanical strength of the proniosome gel as compared to Span® 80. In Fig. 14, the addition of Tween® 20 did not impact the gelation ability of Span® 60 and a semi-solid proniosome gel was successfully formed.

[00172] The mechanical strength of the various proniosome gel formulation was evaluated by observing the flow behaviour of various proniosome gel formulations at different temperatures. 2

mg of the various proniosome gel was heated at a stepwise temperature increment of 5 °C, starting from room temperature at 25 °C until the temperature of 40 °C. The samples were heated for 1 minute. At different temperature, proniosome gels were observed for their transformation into a viscous liquid state and a snapshot of the proniosome gel was taken for further analysis.

5 [00173] As shown in Fig.15, when 40 mg of Span® 80 was added into Formulation A, it was observed that the proniosome gel had started to flow when the sample was heated at 30 °C.

[00174] At 35 °C, the proniosome gel appeared to transform into viscous liquid and it flowed towards the bottom of the microtube. This suggests that 40 mg of Span® 80 had sufficiently reduced the stiffness of the gel when exposed to a heating temperature close to the skin surface temperature of about 32 °C, thus implicating its suitability for dermal applications. Conversely, minimal visible changes were observed when an equal amount of Tween® 20 was added into Formulation A and was heated at 35 °C. The proniosome gel had partially flowed, with the gel smearing along the walls of the microtube instead of flowing down completely towards the bottom. This may indicate that Span® 80 is more effective in reducing the mechanical strength of the gel as compared to Tween® 20. Hence, this demonstrates the necessity of adding Span® 80 to effectively reduce the mechanical strength of the gel.

Comparative Example 2: Comparisons between Formulations of Varying Concentrations of Components

[00175] Varying concentrations of the components of the proniosome gel were also tested when processed according to the method as described above. As shown in Fig. 16, the percentage mass ratio (w/w%) of Span® 60, isopropyl alcohol and phosphate buffer saline solution was varied separately in each sample by fixing the percentage mass ratio of two components while increasing the percentage mass ratio of the third component from 10% to 90% w/w and the proniosome gel formulation was prepared as described herein. The resultant mixtures were cooled to room temperature and photographic images of the samples were taken to observe the physical transition of the coacervate. The physical appearance of the coacervate was plotted accordingly in the phase diagram.

[00176] The first essential step was to form the proniosome gel by optimizing the main ingredient which was Span® 60, IPA and PBS. When the coacervate was formed, it demonstrated that the proniosome gel was successfully formed and the range of concentration required for all the 3 main composition was investigated. Subsequently, with the optimal concentration, Span® 80 and Tween® 20 were added to optimize the mechanical strength and the HLB value of the gel.

Thus, the overall composition as indicated above was normalized against the entire mass of the composition in the proniosome gel (i.e. Surfactants + cholesterol + IPA + PBS and berberine).

[00177] The ternary diagram represented the composition of the proniosome gel containing Span® 60, PBS, and IPA. The region that was highlighted in dashed line in the middle of the ternary diagram corresponds to the coacervate. The coacervate was observed as a semi-solid with creamy whitish colour and it appeared at the top layer of the mixture. The percentage mass ratio of each component was observed to be within the range of (i) Span® 60: 20% to 60% w/w (ii) IPA: 10% to 40% w/w (iii) PBS: 20% to 50% w/w. The optimal composition of the Span® 60-based proniosome gel (Formulation (A)), fell within the coacervate region, with 31% w/w of Span® 60, 38% w/w of IPA and 31% w/w of PBS. Thus, Formulation (A) was selected as a starting point for further optimisation.

[00178] The construction of the ternary diagram had demonstrated the effect of each component in enabling the formation of a gel. The presence of IPA in proniosome gel was observed having dual roles, with its first role acting as a medium in dissolving the non-ionic surfactants under high temperature. The second role of IPA was acting as a dehydrating agent to remove excess water from the mixture when it was cooled to room temperature, and this facilitated the formation of the coacervate. It was confirmed by experiments assessing the impact of increasing concentrations of IPA on the proniosome gel formulation. As the mass percentage of IPA gradually increased, it caused a stronger dehydrating effect on the mixture and it enhanced the interaction among Span® 60 molecules, which led to precipitation at IPA values above 40% w/w. When the mass percentage of Span® 60 increased, the volume of coacervate was observed to gradually increase. This may suggest that there is an increasing amount of niosomes being formed in the gel, which led to an increased volume of coacervate. This suggestion can be at least partially substantiated by the decreasing volume of the diluted liquid component observed at the bottom of the coacervate. The decreased volume of the diluted liquid component can be attributed to the entrapment of the aqueous solution within the hydrophilic core of niosomes. However, when the mass percentage of Span® 60 increased beyond 60% w/w, the surfactants were unable to be dissolved in the limited amount of IPA and they appeared as a waxy solid powder, similar to their original form.

[00179] Concurrently, the volume of coacervate gradually decreased when the percentage mass ratio of PBS increased. At the same time, the volume of the diluted liquid component at the bottom of the coacervate increased, and the solution appeared opalescent. Beyond the percentage mass ratio of 50% w/w of PBS, no coacervate was formed and the mixture appeared turbid. The turbidity was attributed to the presence of niosomes suspension while increasing the aqueous PBS

component reduced the percentage mass ratio of IPA and this reduced its dehydrating effect. Thus, the coacervate was not formed at a higher percentage mass ratio of PBS. Taking all the information together, this study demonstrated the delicate balance among the different components when forming the coacervate and it helped in guiding the subsequent optimisation of the proniosome gel according to the boundaries observed in the ternary diagram.

[00180] Thus, based on the ternary diagram in Fig. 16, it was noted that the suitable concentration of S60 was about 10 weight% to about 65 weight% based on the total weight of the composition; that of S80 was about 5 weight% to about 33 weight% based on the total weight of the composition to effectively reduce the overall stiffness of the gel formed; and that of T20 was about 1.25 weight% to about 8.2 weight% based on the total weight of the composition to maintain a suitable HLB value.

Summary of Examples

[00181] A combination of sorbitan oleate (S80) and polyethylene glycol sorbitan monolaurate (T20) in a sorbitan stearate (S60)-based proniosome (Formulation C) enabled a readily hydrated gel to deliver the antiinflammatory agents (e.g. berberine) into the skin, as confirmed by the ex-vivo skin permeation studies. Concurrently, an in-vitro model of OA using primary mouse chondrocytes demonstrated that the release of berberine at a concentration as low as $1 \mu\text{g mL}^{-1}$ was sufficient to restore the production of sulphated glycosaminoglycans (sGAG) to levels comparable to healthy chondrocytes while avoiding the cytotoxic concentrations ($\text{IC}_{50} = 33 \mu\text{g mL}^{-1}$) on skin keratinocytes. In a mouse model of OA, the optimized formulation was able to attenuate inflammation and pain and minimize cartilage degeneration. Taken together, these data demonstrate the feasibility of adopting proniosome gels as a suitable platform to deliver active molecules for the management of osteoarthritis.

Industrial Applicability

[00182] The disclosed composition may be used for dermal delivery of bioactive molecules. This is applicable to industries such as pharmaceutical, cosmetics, medical and healthcare where the composition can be used to deliver an effective concentration of the drug molecule to its target site and offers an alternative mode of drug delivery system to overcome any systemic side effects.

[00183] It will be apparent that various other modifications and adaptations of the invention will be apparent to the person skilled in the art after reading the foregoing disclosure without departing from the spirit and scope of the invention and it is intended that all such modifications and adaptations come within the scope of the appended claims.

Claims

1. A composition comprising:
 - a) a first surfactant having a transition temperature of at least 50 °C;
 - b) a second surfactant having a transition temperature of less than 20 °C;
 - c) a third surfactant having a hydrophilic-lipophilic balance (HLB) value of at least 15;
 - d) cholesterol;
 - e) an active agent;
 - f) a first solvent; and
 - e) a second solvent.
2. The composition according to claim 1, wherein at least one of the first surfactant, the second surfactant or the third surfactant is a non-ionic surfactant.
3. The composition according to claim 1 or 2, wherein said composition is a proniosome.
4. The composition according to any one of the preceding claims, wherein:
 - the first surfactant has a weight percentage in the range of 5.7wt% to 74.8 wt%;
 - the second surfactant has a weight percentage in the range of 2.8 wt% to 72 wt%;
 - the third surfactant has a weight percentage in the range of 0.71 wt% to 69.8 wt%;
 - the cholesterol has a weight percentage in the range of 0.74 wt% to 69.8 wt%;
 - the active agent has a weight percentage in the range of 0.9 wt% to about 70 wt%;
 - the first solvent has a weight ratio in the range of 10 wt% to 79.1 wt%; or
 - the second solvent has a weight ratio in the range of 10 wt% to 79.1 wt%, based on the total weight of the composition.
5. The composition according to claim 4, wherein the first surfactant, the second surfactant, the third surfactant, cholesterol, the first solvent, the second solvent and the active agent have a weight ratio of 24.8: 12.4 : 3.4 : 3.2 : 30.5 : 24.8 : 0.9.
6. The composition according to any one of the preceding claims, wherein
 - (a) the first surfactant is sorbitan monostearate;

- (b) the second surfactant is sorbitan monooleate, sorbitan monolaurate or combinations thereof;
 - (c) the third surfactant is polyoxyethylene (20) sorbitan monolaurate;
 - (d) cholesterol;
 - (e) the active agent is a hydrophobic molecule;
 - (f) the first solvent is selected from the group consisting of methanol, ethanol, propanol, isopropanol, butanol, 1,2-propanediol, 1,3-propanediol or combinations thereof; or
 - (g) the second solvent is selected from the group consisting of water, a phosphate buffered saline or combinations thereof.
7. A method of forming a composition comprising the steps of:
- (a) heating a first surfactant, a second surfactant, a third surfactant, cholesterol, an active agent and a first solvent to form a mixture; and
 - (b) heating the mixture of step (a) with a second solvent and cooling the mixture to form the composition,
- wherein the first surfactant has a transition temperature of at least 50 °C, the second surfactant has a transition temperature of less than 20 °C, and the third surfactant has a hydrophilic-lipophilic balance (HLB) value of at least 15.
8. The method according to claim 7, wherein said heating steps (a) and (b) are undertaken at a temperature in the range of 50 °C to 80 °C.
9. The method according to claim 7 or 8, wherein said cooling of the mixture in step (b) is undertaken at room temperature or until the composition reaches room temperature.
10. A kit comprising:
- a) the composition according to any one of claims 1 to 6; and
 - b) instructions for using the composition in a).
11. The kit according to claim 10, wherein the composition comprises 200 mg to 400 mg of the active agent.

12. An *in vitro* method of delivering an active agent to a target site, comprising the steps of:
 - (a) providing a composition comprising the active agent as described in any one of claims 1 to 6 or as formed according to the method as described in any one of claims 7 to 10; and
 - (b) administering the composition at a first site to allow the active agent to move to or be transported to the target site.
13. The composition according to any one of claims 1 to 6 for use in therapy.
14. The composition according to any one of claims 1 to 6 for use in treating or preventing of a disease selected from the group consisting of inflammation, cancer, infection and combinations thereof.
15. Use of the composition according to any one of claims 1 to 6 in the manufacture of a medicament for the treatment or prevention of a disease selected from the group consisting of inflammation, cancer, infection and combinations thereof.
16. The composition for use of claim 13 or 14 or the use of claim 15, wherein said composition is to be administered dermally or topically 2 to 3 times daily to a patient.
17. The composition for use of claim 13 or 14 or the use of claim 15, wherein the composition comprises 200 mg to 400 mg of the active agent.
18. The composition for use of claim 13 or 14 or the use of claim 15, wherein the inflammation disease is osteoarthritis.