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Spray-by-spray in situ cross-linking alginate hydrogels delivering a tea tree oil microemulsion

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ABSTRACT

In this paper we propose an *in situ* forming ionically cross-linked alginate (Alg) hydrogel delivering a Tea Tree Oil microemulsion (Me_{TTO}) and potentially useful as an advanced dressing for infected wounds. Alg hydrogels were prepared by a spray-by-spray deposition method with the aim to minimize the discomforts during application. From pseudoternary phase diagrams, it was found that proper combination of TTO, water, polysorbate 80 and ethanol gave stable spherical Me_{TTO} with good antimicrobial activity. On this basis, Me_{TTO} at 20% TTO was selected for further inclusion in an Alg hydrogel prepared by alternating sprays of Alg/Me_{TTO} and calcium chloride solutions. Homogeneous dispersion of Me_{TTO} inside cross-linked Alg was assessed by different macroscopic and microscopic methods demonstrating the superior propensity of Me_{TTO} to be integrated in the water-based hydrogel as compared to TTO. Antimicrobial effect of Alg/Me_{TTO} hydrogels on *Escherichia Coli* strains was remarkable, highlighting the potential of the system as bioactive wound dressing.

1. Introduction

Hydrogels are a network of polymers filled with water that may be applied to absorb wound exudates and to protect wounds from secondary infection. Hydrogel dressings can be applied to the wound either as pre-formed solid gels or as liquids that crosslink after application. An *in situ*–forming hydrogel (ISH) is initially fluid at room temperature but becomes a hydrogel *in situ* due to specific conditions, including ionic cross-linking, pH, or temperature change (Ruel-Gariepy and Leroux, 2004). ISHs have some merits over traditional dressings, including conformability without wrinkling or fluting of the wound bed, ease of application, good patient compliance, and comfort. Thus, ISHs are an excellent option as multifunctional wound dressings suitable for superficial wounds, burns, skin grafts and abrasions that cover a large area.

Alginates (Alg) are a family of polyanionic copolymers derived mainly from brown sea algae (Laurienzo, 2010), and are of growing importance in the healthcare and pharmaceutical industry (Boateng et al., 2008). The ability of Alg to form crosslinks in presence of divalent ions has allowed the development of biocompatible hydrogels that helps to maintain the lesion at an optimum moisture content and healing temperature (Peng et al., 2012). Alg, in combination with other biopolymers or active agents, is widely used as base material in film dressings to improve wound healing rate and to prevent burn infection (Brachkova et al., 2011; Dantas et al., 2011). ISHs based on polysaccharides, obtained by alternating spray of polyanionic and polycationic polymers, are gaining interest in wound management since they allow quick formation of highly uniform thin films over a large surface area (Cado et al., 2012; Schlenoff et al., 2000). Alternate polysaccharide deposition on a solid substrate represents an appealing option to give ISHs also in view of simple industrial scale-up (Schaaf et al., 2012).

Integration of bioactive molecules in ISH is rather simple for hydrophilic compounds but much more challenging for hydrophobic or oily actives. Amid actives with a high potential in wound care, Tea Tree Oil (TTO), a natural essential oil steam-distilled from the Australian native plant *Melaleuca alternifolia*, is gaining a considerable relevance. In fact, TTO is a very promising agent for the treatment of dermatologic diseases due to its antimicrobial effects against a wide spectrum of microorganisms (Carson et al., 2006; Pazyar et al., 2013) and its minimal impact on developing

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resistance (Hammer et al., 2012). Furthermore, it has potent activity against many fungi (Hammer et al., 2003, 2004), protozoa (Carson et al., 2006; Mikus et al., 2000), and certain viruses, including herpes simplex and influenza viruses (Carson and Riley, 2001; Garozzo et al., 2011). Besides the well-known antimicrobial activities, TTO has been shown to possess a number of other therapeutic properties, including anti-inflammatory (Hart et al., 2000; Koh et al., 2002; Pearce et al., 2005) and anti-tumor properties (Bozzuto et al., 2011), especially in skin cancer (Greay et al., 2010; Ireland et al., 2012). Hydrogels containing TTO were already tested in a burn wound model and suggested to increase the rate of wound healing (Jandera et al., 2000). Furthermore, it was recently shown that TTO accelerated wound healing in humans (Chin and Cordell, 2013). As all essential oils, TTO is a lipophilic liquid that shows poor miscibility with water-based products, while its low surface tension demands for methods able to provide an adequate barrier to volatilization.

Microemulsions (MEs) have recently emerged as novel delivery vehicles for hydrophobic drugs by different administration routes (Fanun, 2012). MEs appears as an attractive and competitive system due to many benefits such as easy manufacturing, small droplet size (20–200 nm), high thermodynamic stability, and enhanced solubilization of hydrophobic ingredients. In recent years, MEs have been investigated as potential drug delivery vehicles for transdermal and dermal delivery of several compounds, especially hydrophobic, in order to avoid clinical adverse effects associated with oral administration (Shakeel et al., 2012). Furthermore, MEs can be easily incorporated into Alg hydrogels to create composite hydrogels with a controlled release profile (Josef et al., 2013, 2010). Formation of MEs can represent a viable and efficient approach also to increase physical stability of essential oils, protecting them from undesired interactions and increasing their bioactivity. As demonstrated by Donsi et al. (2011, 2012), MEs can enhance the antimicrobial activity of encapsulated essential oils increasing their water solubility and the consequent capacity to interact with cell membranes.

The objective of the present study was to develop antimicrobial *in situ*-forming Alg wound dressings incorporating TTO microemulsions (Me_{TTO}). Alg hydrogels were prepared by a layer-by-layer spray deposition method with the aim to minimize the discomforts, especially during dressing application. After a thorough characterization of Me_{TTO} and Me_{TTO}-loaded Alg hydrogels, their antimicrobial effect was tested.

2. Experimental

2.1. Materials

Pharmaceutical grade alginic acid sodium salt (Alg) extracted from *Laminaria hyperborea* (viscosity 360 cps) and Tea Tree Oil (TTO) were supplied by Farmalabor (Italy). Polysorbate 80 (Tween® 80), calcium chloride dihydrate ($CaCl_2 * 2H_2O$), sodium chloride (NaCl), potassium chloride (KCl), sodium phosphate dibasic (Na_2HPO_4), calcium chloride ($CaCl_2$), and nile red (NR) were obtained from Sigma–Aldrich (USA). Analysis-grade ethanol was provided by Carlo Erba Reagenti (Italy). Yeast extract, Tryptone and Sodium Chloride, used for prepare Luria–Bertani broth (LB) medium, and Agar bacteriological were purchased from Oxoid Ltd. (Hampshire, UK). Streptomycin sulfate was supplied by Applichem (Germany). The spray pump used for the spray deposition (Classic line equipped with SL pump) was kindly gifted by Aptar Pharma (France). The water used throughout this study was depurated and filtered (Milli Q filter).

2.2. Pseudoternary phase diagrams

The boundaries of the ME domains were determined, with the aid of pseudo ternary phase diagrams, for the polysorbate 80:ethanol (surfactant:co-surfactant), TTO and water phase. The titration method was employed for the construction of phase diagrams. Different mixtures of polysorbate 80 and ethanol (1:1, 2:1 and 3:1) were weighed in a dark-brown, screw-cap glass vial, mixed using a magnetic bar on a stirring plate for 1 h and subsequently stored overnight at room temperature. TTO was then added at ratios ranging from 9:1 to 1:9 to different vials. Finally, aqueous phase was slowly added (under vigorous stirring) with a graduated syringe up to clouding of homogenous mixture of oil and surfactants/co-surfactants. Approximately 10 data points were obtained to determine each pseudoternary phase diagram. No attempts were made to completely identify the other regions of the phase diagrams in detail, and these have been described in terms of their visual and external appearance. To graphically show the phase variations of polysorbate/ethanol–water–TTO system, a pseudo-ternary phase diagram was built using Microsoft excel software.

2.3. Preparation of microemulsions

Once the MEs region was identified, TTO was dissolved in a surfactant/co-surfactant mixture previously prepared using a magnetic stirring plate. An oil-in-water ME was prepared by slowly adding water to the oily phase (oil plus surfactants) under continuous magnetic stirring. To prepare the NR-loaded ME, the probe was dissolved directly into TTO at the concentration of 20 μg/mL. All the formulations were prepared at room temperature and tested after 24 h. Resulting MEs were visually inspected and turbidity was measured using a spectrophotometer.

2.4. Characterization of microemulsions

Droplet size and polydispersity index (PDI) of MEs was measured by Dynamic Light Scattering (DLS) using a Zetasizer NanoZs (Malvern instruments, UK). MEs were diluted 1:10 with deionized water prior the experiment to avoid the effect of viscosity and to trim down multiple scattering effects. Particle size and PDI measurements were performed at a scattering angle of 90° and at a temperature of 25 °C.

ME droplet morphology was visualized by Transmission Electron Microscopy (TEM). Samples for TEM analysis were prepared by placing one drop of MEs onto a copper grid. After approximately 1 h, images were captured. TEM analysis was performed with a FEI Tecnai G12 (LAB6 source) equipped with a FEI Eagle 4 K CCD camera (USA) operating with an acceleration voltage of 120 kV.

Viscosity of the formulated MEs was measured using a Brookfield Viscometer (model LVF 69726) supplied with UL-adapter. All the experiments were performed in triplicates at 25° C.

Turbidity of the formulated MEs was evaluated on a UV/VIS spectrophotometer (UV 1800, Shimadzu, Japan) at a wavelength of 502 nm, fitted out with a 1 cm quartz cell (Hellma, Germany). The turbidity was calculated as turbidity × path length = 2.303 × absorbance (Fletcher and Morris, 1995).

The pH of each formulation was evaluate using a Crison basic 20 pHmeter equipped with an electrode 50 10T (Crison, Spain).

2.5. Microemulsion stability studies

2.5.1. Centrifugation study

The formulated MEs were studied for their stability to centrifugation by placing the sample at 13,000 rpm for 30 min (Mikro 20 centrifuge, Hettich, Germany) and observing phase separation, creaming or cracking (if any).

2.5.2. Heating–cooling cycle

This study was performed to check the effect of temperature variations on the stability of MEs. Samples were stored between