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Tracking the transdermal penetration pathways of optimized curcumin-loaded chitosan nanoparticles *via* confocal laser scanning microscopy

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ABSTRACT

Curcumin-loaded chitosan nanoparticles intended for transdermal delivery were successfully prepared, optimized and their fate, interaction and pathway through the skin were tracked. D-optimal response surface methodology was used for the nanoparticles optimization. *Xy* and *z*-stack confocal laser scanning microscopic images were used for the particles tracking after measuring the drug permeation through the skin using Franz diffusion cells. Very small particle sizes in the range of 33.85–199.23 nm accompanied with low PDI values of 0.129–0.536 of the prepared curcumin-loaded chitosan nanoparticles were obtained. TEM images revealed the spherical and non-aggregating curcumin-loaded chitosan nanoparticles. The *ex-vivo* permeation studies have proven the ability of the prepared chitosan nanoparticles to deliver curcumin through the skin reaching fluxes *viz* $5.14 \pm 1.31\ \mu g\,cm^{-2}\,h^{-1}$. The confocal laser scanning microscopy has proven that the appendageal route is the main route of penetration of the prepared nanoparticles and has demonstrated the localization of the chitosan nanoparticles within the hair follicles from which the drug diffuses to deep layers of the skin and beyond.

1. Introduction

The transdermal route has gained much interest for many years of extensive research; thanks to its impressive advantages compared to other routes of drug delivery. Transdermal delivery systems are able to deliver drugs directly to the systemic circulation through the skin barrier, avoiding the hepatic first pass effect, the acidic environment of the stomach, the pH changes, the effect of gastrointestinal enzymes and the fluctuating plasma concentrations associated with the oral route [1–5]. Moreover, transdermal delivery is capable of maintaining a constant and prolonged drug plasma concentration at a predetermined rate, thus reducing side effects and therapeutic failure [1,5]. Additionally, transdermal formulations have a high patient compliance as they are non-invasive, can be self-administered [6] and offer the flexibility to terminate the drug administration through the simple removal of the patch from the skin [4]. However, one major limitation of the transdermal delivery systems is the probable skin irritation or sensitization which may be triggered by the drug itself or the carrier components [7]. In order to overcome this limitation, using a biocompatible and non-antigenic biopolymer, such as chitosan, can be a reasonable approach [8].

Chitosan is a natural cationic polysaccharide that exhibits many remarkable properties such as biodegradability, non-toxicity, hemostatic and antimicrobial activity, bio-adhesive and penetration enhancing properties [9–13]. Many studies have shown that chitosan and its derivatives are capable of significantly enhancing the mucosal drug absorption [14,15]. Chitosan binds to the negatively charged sites on the epithelial cell membranes and tight junctions [16] where its positive charges result in the depolymerization of F-actin and the disbandment of the tight junction protein ZO-1, and hence opening the tight junctions [1].

Chitosan has gained more interest as an effective skin permeation enhancer in different formulations such as transdermal films and membranes [17,18,8], transdermal patches [2], microgels and nanogels [19,20], nanofibers [21], and nanoparticles [22,1,23,3,4,24,25]. The structural composition of the *stratum corneum*, the main barrier against skin permeation [26], is very different from the epithelial cells. However, the *stratum corneum*

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has fixed negative charges in the tight junctions which are similar to those of the epithelial cells [18,27]. Taveira et al. [28] reported that chitosan improved drug diffusion to deeper skin layers as it appeared to interact with the skin negative charges. Other studies concluded that chitosan increases the water content in the *stratum corneum* [23,27], changes the secondary structure of keratin [27,29], decreases cell membrane potential and enhances cell membrane fluidity [27].

Polymeric nanoparticles are used to enhance the bioavailability, absorption and penetration of drugs [30], protect drugs from premature degradation and control the rate of drug release [3]. Previous studies have shown the potential of nanoparticles for transdermal delivery [22,31,23,3,4]. It has been demonstrated that nanoparticles of a particle size less than 500 nm penetrate better through the skin [32]. This small size probably ensures a close contact with the *stratum corneum* and increases the drug permeation into the skin [3,25]. The follicular route appears to be the key penetration pathway for nanoparticulate drug delivery systems.

It was believed for long that the contribution of the appendageal route in the transdermal penetration is not significant compared to the intercellular route [33], as the skin appendages occupy only approximately 0.1% of the skin surface area [34]. However, it was not taken into consideration that the hair follicles invaginations extend deeply into the dermis, significantly increasing the actual surface area available for permeation [35,36]. In addition, the hair follicles are surrounded by a rich network of blood capillaries [37,38], as well as the hair follicle stem cells and dendritic cells which are important for regenerative medicine and immunomodulation respectively [39–41].

Particulate systems were reported to penetrate deeper and very efficiently through the hair follicles [31,42–51] compared to the non-particulate systems [44,52,46,47,51]. In addition to this, the hair follicles represent efficient long-term reservoirs for topically applied substances [44,53,45,50], in contrast to the *stratum corneum*, from which drug depletion can occur through washing, textile contact or continuous desquamation [54]. Interestingly, the storage time of the particles was found to increase by up to 10 days, which is longer than the 4 days reported for the non-particulate systems [55,53].

Curcumin is a multi-purpose therapeutic agent with a proven efficacy in the prevention and treatment of various tumors [56–60]. It modulates multiple molecular targets involved in the regulation of cellular proliferation, apoptosis and angiogenesis [61–64]. However, the physicochemical and pharmacokinetic properties of curcumin render its formulation and systemic delivery a difficult task to accomplish. This molecule is very poorly water soluble, photodegradable, hydrolysable in alkaline conditions, liable to rapid metabolism and elimination and of a short half-life [65,58]. Hence, it suffers a low oral bioavailability resulting in a limited *in-vivo* efficacy. The transdermal route offers a very reasonable approach to enhance the systemic delivery of drugs with diminished oral bioavailability and particularly with short half-lives such as curcumin. Moreover, the molecule of curcumin has its own fluorescence, therefore, it would be easier to track its pathway within the skin using confocal imaging.

Modeling the fabrication of drug-loaded nanoparticles remains a difficult and essential task at the same time. And to accomplish this target, different experimental designs that are capable of proposing information-rich experimental points are usually utilized. Among the different types of experimental designs, the response surface methodology (RSM) has become the standard approach used for optimization purposes, both in laboratory and industry. RSM is mostly concerned with generating a polynomial function, usually a first or a second-order model. These designs allow us to estimate the shape of the response surface we are investigating and therefore, they are termed response surface designs [66]. RSM optimizes multiple variables by systematic variation of independent variables in a well-designed experiment with minimum number of experiments thus depicting a complete picture of variation of the product/process responses as function of formulation variables. The RSM optimization process involves three main steps: (1) performing statistically designed experiments, (2) generating mathematical models, and (3) predicting the response and validating the adequacy of the model [67]. Among the available and successful response surface methodologies are the D-optimal designs. These designs were proven to more efficiently cover all the design space than the other designs and hence generate more accurate and less biased mathematical models [68]. Besides, they are considered more robust to constraints [69].

The objective of our present work is to optimize the formulation of curcumin loaded chitosan nanoparticles and investigate the potential of these nanoparticles for enhancing the transdermal delivery of curcumin. Mathematical models for the studied factors influencing the nanoparticles production were generated and validated. Formulations were selected and characterized. *Ex-vivo* permeation experiments were carried out and confocal imaging was performed to track the penetration pathways of the curcumin chitosan nanoparticles through the skin.

2. Materials and methods

2.1. Materials

Low molecular weight (LMW) chitosan (ChitoClear® fg 95LV, specifications: viscosity of 1% solution in 1% acetic acid <25 cps, deacetylation degree >95%) derived from shrimps was a kind gift from Primex® (Siglufjordur, Iceland). Curcumin (assay ≥94% (curcuminoid content), ≥80% (curcumin)), sodium tripolyphosphate (TPP), glacial acetic acid and fluorescein isothiocyanate (FITC) were purchased from Sigma-Aldrich Chemical Co. Ltd. (St. Louis, USA). Polyethylene 80 sorbitan monooleate (Tween 80®) was purchased from Merck Co. (Darmstadt, Germany). Trehalose, methanol (HPLC grade) and absolute ethanol (analytical grade) were purchased from Fisher-Scientific (Loughborough, UK). Sodium hydroxide and hydrochloric acid were purchased from Adwic, El-Nasr Pharmaceutical Co. (Cairo, Egypt). Nanosep® centrifuge tubes fitted with an ultra-filter with a molecular weight cut off of 100 kDa were purchased from Pall Life Sciences (USA). Spectra/Por® dialysis membrane, 12,000–14,000 molecular weight cut off was purchased from Spectrum Laboratories Inc. (Rancho Dominguez, Canada). Nalgene® Millipore filters of pore sizes 0.2, 0.45 and 0.8 μm were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Phenomenex® C_{18}, 5 μm, 50 mm × 4.6 mm column was purchased from Phenomenex (Torrance, CA, USA).

2.2. Preparation of curcumin loaded chitosan nanoparticles

Chitosan purification was carried out as previously described in literature [70,68]. Afterwards, chitosan solution of 0.1% (w/v) concentration in 1.5% (v/v) glacial acetic acid was prepared. Stirring for 30 min (Heidolph, Schwabach, Germany) then vacuum filtration through 0.45 μm filter were performed. A solution of 10 N NaOH was used to adjust the pH of the chitosan solution to 4.5, 5.0 and 5.5. Tween 80 was added to the chitosan solution with 0.20%, 0.35% and 0.50% (v/v) concentrations to enhance the solubility of curcumin [71]. TPP solutions of 0.0830%, 0.0625% and 0.0500% (w/v) were prepared and filtered through 0.20 μm filter. A solution of 1000 μg/ml curcumin in methanol was prepared.

The nanoparticles were prepared using the ionic gelation method [72] with slight modifications for the drug loading. Curcumin solution was added with the desired amount (0.50, 0.75 or