Short communication

Retinyl palmitate flexible polymeric nanocapsules: Characterization and permeation studies

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\textbf{ABSTRACT}

Polymeric nanocapsules with elastic characteristics were prepared by the pre-formed polymer interfacial deposition method. The system consists of an oily core of retinyl palmitate with Span 60 and a polymeric wall of poly(D/L-lactide) (PLA). A narrow size distribution (215 nm, P.D.I. 0.10) was showed by dynamic light scattering (DLS) analyses. Particle deformability was observed by transmission electron microscopy (TEM) images and permeation of the particles through two superposed membranes of smaller pore diameters. Permeation studies were achieved using plastic surgery abdominal human skin by Franz diffusion cell. Retinyl palmitate permeates into deep skin layers. Besides, a PLA fluorescent derivative conjugated with Nile blue dye by an amide covalent bound was additionally obtained. Permeation profile of the nanocapsules with the fluorescent polymer was evaluated by confocal laser scanning microscopy (CLSM). The CLSM showed that nanocapsules were distributed uniformly, suggesting that the permeation mechanism through skin is intercellular. Thus, the use of these nanocapsules may be a feasible strategy to enhance the permeation of actives into the skin when delivery to deep layers is aimed.

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1. Introduction

Nanostructures in drug delivery have been widely used in recent years because they cannot only trigger a sustained release and protect labile drugs from degradation, but also make passage through some biological barriers feasible [1,2]. For dermatologic purposes, biodegradable polymeric nanoparticles have commonly been used to enhance the permeation of drugs through skin [3,4]. Polymeric nanoparticles can be classified as nanospheres (matrix system) or nanocapsules (reservoir system). One of the main advantages of nanocapsules is a higher drug loading than nanospheres. Their shell generally consists a biocompatible and biodegradable polymer while their core commonly employs an oil dissolving the drug [4–6].

The skin has a brick-and-mortar-like structure so permeation mechanisms may be intercellular (through ‘mortar’) or intracellular (through bricks). Another potential mechanism is via appendices, which is basically by pilosebaceous follicles that represent only around 1% of the total skin area or by narrow skin pores. The passage through the pores is influenced by a suitable transdermal gradient [7–10].

The mechanism of permeation through the skin is still not fully investigated [3,4,11]. Some authors have suggested the formation of a uniform film that inhibits the transepidermal loss of water and upholds a uniform drug release [12–14]. Others have suggested that nanoparticles can reach deeper layers in the skin [8–11,15]. Actually, both mechanisms are possible depending on the physicochemical characteristics of the particles such as rigidity, hydrophobicity and size. Polymeric nanoparticles principally show characteristics of film formation whereas elastic liposomes can permeate skin deeply [8–10,12–14].

By using confocal laser scanning microscopy (CLSM) of single stained nanoparticles, poly(lactide-co-glycolide)-borne flufenamic acid as a model was investigated after topical applications. The transdermal delivery of flufenamic acid was improved by using...
nanoparticles whereas the carrier remained on the skin surface [13]. Multiphoton microscopy using dual color labeling was used to distinguish between carrier, nanoparticles of PLGA covalently bound to fluorescein, and a drug model (Texas red). This study showed that only the active principle penetrates into the skin layers [14]. Self-assembled vesicles of poly(caprolactone)–poly(ethylene glycol)–poly(caprolactone) were investigated by TEM showing flexible vesicle characteristics. The authors also observed a deeper release of Nile red as a model drug encapsulated in this system, which suggests their use for targeting melanomas and basal cell carcinoma [15]. A comparison between different systems of nanocapsules, nanospheres and nanoemulsion in the encapsulation of nimesulide showed the polymer content carriers, nanocapsules and nanospheres, have higher stratum corneum drug retention, while a drug in nanoemulsions goes straightforwardly to deeper skin. The system of nanocapsules presented the highest nimesulide penetration [16].

Elastic characteristics of nanocarriers were first evaluated for liposomes, in which the possibility to go across pores of smaller diameters with recovery of the initial configurations suggested the elasticity of the system [17]. A lot of deformable liposome systems have been investigated since then, such as ultradeforable cationic liposomes, for delivery of small interfering RNA into human primary melanocytes [18], as well as, liposomes for sustained delivery of rizatriptan, an antimigraine drug [19], and propanolol hydrochloride, an anti-hypertensive agent [20].

Retinyl palmitate oil is the most stable form of vitamin A and plays an important role in cellular differentiation and carciogenesis prevention [21]. Furthermore, it is largely employed in anti-ageing formulations.

In this work, we propose a novel flexible polymeric nanocapsule with a retinyl palmitate core and poly(D,L-lactide) (PLA) shell as carrier of drugs or simply as a reservoir of vitamin A. The main advantage is that these capsules should pass easily though biological barriers due to deformability characteristics.

2. Materials

The materials employed were: poly(D,L-lactide) (PLA) with molar mass of 75–120 kDa, retinyl palmitate, polyoxyethylene-polyoxypropylene-polyoxyethylene (Pluronic F68), butyldihydroxytoluene (BHT), Nile blue hydrochloride (NB) and N-hydroxy succinimide (NHS), all purchased from Sigma-Aldrich (St. Louis, MO, USA), octaoxyethylene laurate ester (PEG-8L), a gift from Lipo Chemicals (Paterson, NJ, USA), Tinogard Q from Ciba (Basel, Switzerland), sorbitan monostearate (Span 60F) and the ethoxylated sorbitan monooleate, 20 EO (TWEEN 80), a gift from Oxiteno (São Paulo, Brazil). Dicyclohexylcarbodiimide (DCC) was from Fluka (Bucks, Switzerland), Dichloromethane (DCM), acetonitrile (ACN), acetone, ethyl acetate, and ethanol (EtOH) were of analytical grade and were obtained from Merck (Darmstadt, Germany). Carbopol 940® was acquired from B.F. Goodrich (São Paulo, Brazil). All solvents and chemicals were used without further purification.

3. Methods

3.1. Nanocapsules preparation

The nanocapsules were prepared by a modification of the preformed polymer interfacial deposition method (nanoprecipitation) [22]. Briefly, the organic phase containing 50 mg PLA, 1% BHT, 0.3% (w/v) Span 60F, 150 mg retinyl palmitate, 15 mL acetone and 5 mL ethanol were poured into 40 mL aqueous phase (1% (w/v) Pluronic F68, 1% (w/v) Tween 80 and 0.1% (v/v) Tinogard Q®) under magnetic stirring. The water phase temperature was 37 °C during the addition of organic phase. The organic solvents were eliminated and the final suspension was concentrated to 10 mL under reduced pressure with a rotary evaporator.

3.2. Measurement of particle sizes and zeta potential

Size distributions, polydispersity (P.D.I.) and zeta potential were determined in the Zetasizer Nano ZS equipment (Malvern Instruments, Worcestershire, UK). The measurements were made in a KCl 1 mmolL⁻¹ diluted suspension at 25 °C.

3.3. High performance liquid chromatography (HPLC) analysis

Retinyl palmitate in the samples was evaluated by HPLC analysis in a Shimadzu equipment (detector SPD-M20A), C18 Nova Pak Waters column, 3.9 mm × 150 mm. The mobile phase consisted of MeOH 100%. The flow-rate was 1.0 mL min⁻¹; the temperature was 25 °C and the detection at 325 nm. Linear response curves were constructed from the limit of determination (0.2 µg mL⁻¹) to 10.0 µg mL⁻¹ (r > 0.999). The retinyl palmitate retention time was 16.2 min. Accuracy and precision values were calculated and they were below 2% (R.S.D.). The limit of detection of the method was 0.1 µg mL⁻¹. The selectivity of the assay was confirmed by the individual analysis of blank samples from the nanospheres (without retinyl palmitate), tape, skin, and from the receptor compartment of the Franz diffusion cells.

3.4. Encapsulation efficiency

Retinyl palmitate quantification was carried out by HPLC as described above. The total drug in the suspension was extracted by the addition of 100 µL of samples in 9.9 mL of ACN. The retinyl palmitate encapsulation efficiency was calculated by ultracentrifugation/filtration, in which 450 µL of suspension in a membrane (Millipore Microcon 100 kDa) was centrifuged for 10 min at 8000 × g. The calculation of the encapsulation efficiency was [(total drug – free drug)/total drug] × 100.

3.5. Morphological analysis

Sample morphologies were investigated by transmission electron microscopy (TEM). A diluted suspension drop was placed on a copper grid (400 mesh) coated with Formvar/carbon. After 2 min, the liquid was removed by capillarity with a piece of paper. A drop of 1% uranil acetate was added and, after 1 min, its liquid was removed by capillarity. The samples were analyzed in a Carl Zeiss CEM902 (Oberkochen, Germany), operating at 80 kV.

3.6. Elasticity measurements

The elasticity of the samples was measured by the method described for elastic liposomes [9,23,24]. Briefly, the nanocapsule suspensions were extruded through two crossed polycarbonate filters (Millipore, USA) of 50 nm pore diameter and 13 mm diameter using a stainless steel pressure holder at 2.5 × 10⁵ Pa and 6.0 × 10⁵ Pa. The temperature was kept at 25.0 ± 0.5 °C using a circulating water bath. Size distribution and morphologies were compared before and after the permeation through the membranes.

3.7. Skin permeation experiments using Franz diffusion cell

Human abdominal skin was donated from plastic surgery. The subcutaneous lipid tissue was removed immediately after the surgery and the skin was stored frozen for up to 1 month before
use. The Franz diffusion cell used was with nominal area of 2.01 cm² and receiver compartment with 7.0 mL capacity. The epidermal side was exposed to the donor chamber and the dermal side was bathed with degasified PBS, containing Tween 80 5 wt.% in the receptor chamber. The receptor medium was kept at 37 °C and under magnetic stirring. Fresh suspensions of retinyl palmitate nanocapsules incorporated in 0.5 wt.% Carbopol 940® gel were carried to the donor compartments, following an incubation time of 24 h (HPLC determination) or 4 h (CLSM investigation).

### 3.8. Drug skin extractions

At the end of the incubation time in Franz diffusion cell, the surface of the skin was 3-fold washed with PBS and gently dried with a cotton swab. The amount of retinyl palmitate in the receptor chamber was analysed by HPLC as described in Section 3.3. The retinyl palmitate in the stratum corneum (SC) was evaluated after tape-stripping 20 times using Scotch Tape 750 (3M, St. Paul, MN). The retinyl palmitate was extracted from the tape in 5 mL of methanol at 40 °C in ultrasonic bath and vortexed 4 times for 30 s in a period of 60 min. The remaining tissue was sliced in small pieces in another vial with 5 mL of ethyl acetate and extracted by the same procedure. Samples were filtered through membranes (0.45 µm Millipore) and analysed by HPLC (Section 3.3). Each result was expressed as the mean values ± standard deviation for n = 6 Franz diffusion cells [25].

### 3.9. Preparation of Nile blue-labelled PLA

To prepare a polymeric fluorescent derivative, the amino group of Nile blue was covalently bound to the end carboxylic group in PLA [26,27]. PLA (0.01 mmol) and NHS (0.16 mmol) were dissolved in DCM (10 mL). The obtained solution was cooled in an ice bath and DCC (0.15 mmol) in DCM (2 mL) was added. The reaction mixture was maintained under argon atmosphere, magnetic stirring and cooling for 2 h. At the end of this period, Nile blue (0.17 mmol) in ACN (4 mL) was added at room temperature, followed by incubation under argon atmosphere and magnetic stirring for 24 h. The solvent mixture was evaporated under reduced pressure in rotary evaporator and the solid re-suspended in chloroform (10 mL). Then, the organic phase was separated and washed 5 times with 10 mL of HCl (5 mM) and 5 times with 10 mL of deionised water. The polymer from this latter chloroform layer was precipitated twice over cooled ethanol (50 mL) and washed with water. The pellets were lyophilized and stored at −20 °C until use. The fluorescent polymeric derivative was characterized by UV–vis absorption spectroscopy, fluorescence spectroscopy and gel permeation chromatography (GPC) (data not shown).

### 3.10. Confocal laser scanning microscopy (CLSM)

At the end of the incubation time in Franz diffusion cell, surface of the skin was washed with PBS and gently dried with a cotton swab. The skin was immediately sliced with a sharp blade in rectangular pieces, embedded in physiological solution and placed, epidermis side down, on glass coverslips. The samples were then microscopically examined without additional tissue processing using an inverted laser scanning confocal microscope LSM 510 META (Carl Zeiss, Jena, Germany), with an oil objective of 63 × and numerical aperture of 1.4. The pinhole device was used allowing 1 unit airy for each fluorescent probe. Depth scanning was evaluated around 100 µm. The Nile blue was excited with a HeNe laser (excitation = 546 nm) and light emission was detected using a bypass filter (emission = 590–650 nm). The retinyl palmitate was excited with a 740 nm multiphoton Coherent laser Ti-sapphire (excitation around 370 nm) and light emission was detected at 419–548 nm. The images were overlaid (Zeiss LSM confocal software) to provide dual-coloured images. In separate CLSM experiments, skin samples were examined in the absence of the fluorescent probes. To visualize the distribution of fluorescent nanoparticles, confocal images were first obtained in the xy-plane (i.e., parallel to the plane of the skin surface). The skin surface (z = 0 µm) was defined as the imaging plane of brightest fluorescence with a morphology characteristic of the stratum corneum surface. To generate an xz-section, a horizontal line was “drawn” across a region of interest in the z = 0 µm-xy-plane and then “optically sliced” through the digitized image data of the successive xy-sections to generate xz-planar optical cross-sections. All images presented in the figures are necessarily representative of at least three scans and were obtained with the same optical aperture, lens and scan speed. Attempt to capture what was typically observed under each control and treatment condition was considered.

### 3.11. Statistics

Results were expressed as mean values ± standard deviation. The comparisons between groups were done using one-way analysis of variance (ANOVA) followed by Bonferroni’s modified test. P < 0.05 was taken as significant.

### 4. Results and discussion

#### 4.1. Nanocapsules characterization and mechanism of deformability

The nanocapsule average diameter and zeta potential were 215 nm (P.D.I. = 0.1) and −14.9 ± 5.0 mV, respectively. The zeta potential values were in the range expected for nanoparticles from polyesters, which are slightly negative due to some hydrolyzed groups in the particle surface [28].

Fig. 1 shows a TEM micrograph, which indicates that the nanocapsules present deformability when dried on the sample holder. The mechanism of deformability can be understood by capillary adhesion forces (Fig. 2). During the drying process over the sample holder, the film of aqueous phase that wet the particles shrinks and the radius of the meniscus (capillary) between the particles diminishes [29]. According to the Young–Laplace equation (Eq. (1)) [30], there are differences of pressure (∆P) inside the
meniscus, which is dependent on the radius ($R$) and the interfacial tension ($\gamma$). Thus $\Delta P$ increases with the decrease of the radius formed by the aqueous film.

$$\Delta P = \frac{2\gamma}{R} \quad (1)$$

Fig. 2 illustrates this mechanism, in which $\Delta P_2 > \Delta P_1$. Thus the capillary adhesion force is enough to cause the deformation of the nanocapsules. To prove this mechanism, we can observe that the particles do not show deformities when dried without the neighborhood, maintaining spherical morphology.

Rastogi et al. found a similar result in the self-assembled system of poly(caprolactone)–poly(ethylene glycol)–poly(caprolactone) co-polymer. These vesicles showed average sizes of 122 ± 20 nm and wall thicknesses of with 25 nm. The authors suggested that the deformability nature of this system increases its permeation through skin [15].

### 4.2. Elasticity measurements

As a result of the particle deformability under capillary forces, measurements of their possible elasticity were investigated as described for elastic liposomes [9,23,24]. The human skin has pores with average diameters of 30 nm and transepidermal pressure around 2.5 × 10^5 Pa. Thus such assay also simulates skin permeation in vivo.

The nanocapsules without an additional surfactant are retained on the membranes, most probably due to capillary repulsion forces between membrane pores and particles. The addition of PEG 8L (0.2%, m/v) increases the wettability of pores and leads to the passage of the particles, as observed for elastic liposomes as well [31]. Therefore, the permeation of the nanocapsules through narrow pores was possible (Table 1). Both extrusion experiments, under 2.5 × 10^5 and 6.0 × 10^5 Pa, resulted approximately in the same average diameters with fluxes of 0.20 g h^{-1} cm^{-2} and 0.38 g h^{-1} cm^{-2}, respectively.

Honeywell-Nguyen et al. have investigated the mechanism in which PEG-8L gives to liposomes this elastic characteristic. Elastic vesicle pre-treatment and elastic vesicle co-application were investigated. Besides, a comparison was made between the drug transports from two elastic vesicles formulations with different entrapment efficiencies (99.8 ± 0.02 and 21.1 ± 9.6%). In these results, pre-treatment with PEG-8L did not result in permeation enhancement, which means that PEG-8L does not change the stratum corneum structure. The elastic vesicles are 5-fold more efficient than PEG-8L micelles in permeation through skin. Higher encapsulation efficiencies resulted in deeper drug permeation. This means that the free drug has lower permeation efficiency than the encapsulated one. Thus, the high permeation efficiency was indeed because of the elastic characteristic of the vesicles [31].

It is worth mentioning that in our system, PEG-8L was initially used above its c.m.c. but the extruded sample was diluted for DLS analyses below its c.m.c. Therefore, the diameter measurement measured is indeed the nanocapsules diameter distribution.

Because large particles scatter much more light than small ones, the results of DLS are generally better expressed by number instead of intensity or volume in polydisperse systems [32]. Fig. 3 shows diameter distribution by intensity and by number with respect to Table 1. The graph shows that particles with diameters close to the average diameter pass across the membranes while particles with smaller diameter, probably more rigid, as well as particles with larger diameter are retained on the barrier. Fig. 4 shows the TEM

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<td>Extrusion of nanocapsules through polycarbonate crossed membranes of 50 nm pore sizes.</td>
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images for the permeated (extruded) and retained particles, which corroborate with the data obtained by DLS analyses. The images show particles of diameters close to the initial average diameter for the extruded samples, which are the particles with diameters between around 100 and 300 nm. The sample that permeates the membranes recovers a configuration close to the initial one, that is, a spherical morphology, suggesting an elastic characteristic to this system. We suggest that the higher diameter of particles after filtration may be associated with a higher surface area since they are not totally spherical anymore. Further, some particles can undergo coalescence during extrusion. The particles retained on the filter are mainly the ones with the smallest and the largest diameters, below approximately 100 nm and above 300 nm, respectively. These results corroborate with Fig. 1, which shows that some small particles are not deformable. We also suggest that particles larger than 300 nm are not flexible enough to pass across the membrane.

4.3. Skin permeation studies: HPLC

Fig. 5 shows the result after 24 h of permeation in Franz diffusion cell. The nanocapsules permeated through deep layers into the skin, since the amount of drug in the dermis plus the epidermis without stratum corneum presented the highest amount. In addition, the drug reached the receptor chamber. The high permeability of the nanocapsules is probably because of the system deformability characteristics.

The skin is an effective barrier against external medium, in which the stratum corneum plays the function of main barrier [33]. When
molecules reach the end of stratum corneum, it is expected to permeate through deeper layers of epidermis and dermis and the molecule can reach systemic absorption. Depending on the site of action in the skin for dermatological or cosmetic products, it is necessary that the bioactive reaches viable cells in the skin (viable epidermis or dermis).

Generally, retinoids presents low permeation into skin [34]. Thus, the nanocapsules are a promising system for retinyl palmitate delivery. Conversely, SLN containing retinyl palmitate showed an improvement of the penetration barrier property instead of drug delivery to the deep layers [35].

4.4. Skin permeation profile: CLSM

In this study, the skin permeation profiles of the samples were analysed by CLSM, obtaining dual-labelled images by sequential excitation. Nile blue labelled-nanocapsules (shell on the system) were observed in red, while the green fluorescence corresponded to the retinyl palmitate from nanocapsules samples (Fig. 6). Skin auto-fluorescence was discounted by analysis of samples previously incubated with non-functionalized nanospheres (in the absence of fluorescent probes) at the same experimental conditions.

A major advantage of CLSM is that the tissue can be optically sectioned, thus enabling the depth-dependent distribution of the fluorescent probes to be visualized, without tissue fixation and/or sectioning [36]. The stratum corneum, main limiting barrier in transdermal paths, comprises 15–20 layers of corneocytes. Its thickness can reach 40 μm hydrated and 10–15 μm dried [37]. Nanocapsules presented uniform permeation into the skin. Moreover, a deep permeation, around 30 μm, of both the carrier and the active drug was observed after 4 h of incubation (Fig. 6). However, a co-localization of red (PLA-Nile blue) and green (retinyl pamitate) fluorescence was not observed. In the experimental conditions used in this study, the retinyl palmitate in the core exhibited quenching effect. However, using a lower retinyl palmitate amount, the red fluorescence from the polymer functionalized is not detectable (data not shown). Then, the green fluorescence was most probably due to the non-encapsulated retinyl palmitate. The free retinyl palmitate was found on the surface of skin while the polymer went to deep layers. The free retinyl palmitate most probably from release and the polymer (carrier) were found in deep layers. Thus, the nanocapsule system was better characterized by the red fluorescence of the polymeric wall. This uniform permeation suggests that the main mechanism of nanocapsules permeation is intercellular, through thin pores of the skin.

Previous results indicated that polystyrene nanoparticles preferentially accumulated and permeated the skin through the follicular route [37], although a uniform release of the active through the skin has also been reported, which was dependent upon the formulation nature and particle size [12,13]. Nanospheres of poly(D-lactide-co-glycolide) covalent-bounded with fluorescein and Texas Red as drug model were investigated by multiphoton microscopy [14]. No penetration of the particles were found, the fluorescein remained on the skin surfaces, while Texas Red was released and accumulated in the stratum corneum down to approximately 20 μm after 5 h. These results mean that the type of nanocarrier is crucial for drug skin permeation.

Our results demonstrated a deep dermal permeation of the polymer carrying the active drug (retinyl palmitate). It is worth mentioning that there is no rule to predict the permeation of nanoparticles, because it depends on the unique characteristic of each system, such as surface properties, rigidity and particle diameters. In our system, we can suggest that the deep permeation may be related to nanocapsules affinity for the stratum corneum, as well as, to their flexible characteristics.

5. Conclusions

A polymeric nanocapsule system of retinyl palmitate core and poly(D,L-lactide) shell with low polydispersity and flexibility characteristics was obtained. The quantification by HPLC showed that,
after permeation in Franz diffusion cell, retinyl palmitate is found in the stratum corneum, in deeper layers into skin and in the receptor chamber. The deeper and uniform permeation in the skin was confirmed by CLSM analyses. These results make feasible the use of this nanocapsule system to deliver retinyl palmitate, which might be associated with other actives, into deep layers of the skin.

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