Biodegradable nanoparticles containing benzopsoralens: An attractive strategy for modifying vascular function in pathological skin disorders

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Abstract

Psoralens are often used to treat skin disorders such as psoriasis, vitiligo and others. The toxicity and fast degradation of these drugs can be diminished by encapsulation in drug delivery systems (DDS). Nanoparticles (NPs) containing the benzopsoralen (BP) (3-ethoxy carbonyl-2H-benzofuro[3,2-e]-1-benzopiran-2-one) were prepared by the solvent evaporation technique, and parameters such as particle size, zeta potential, drug encapsulation efficiency, and external morphology were evaluated. The analysis revealed that the NPs are spherical and possessed a smooth external surface with diameter of 815 ± 80 nm, they present low tendency toward aggregation, as confirmed by their zeta potential (+17.3±2.9 mV) and the encapsulation efficiency obtained was 74%. The intracellular distribution of NPs as well as their uptake by tissues was monitored by using laser confocal microscopy and transmission electron microscopy. The use of benzopsoralen in association with ultraviolet light (360 nm) revealed morphological characteristics of cell damage such as cytosolic vesiculation, mitochondria condensation, and swelling of both the granular endoplasmic reticulum and the nuclear membrane. The primary target of DDS and drugs in vascular system are endothelial cells and an attractive strategy for modifying vascular function in various pathological states of skin disorders, cancer and inflammation. The result presented in this work indicates that PLGA NP could be a promising delivery system for benzopsoralen in connection with ultraviolet irradiation therapy (PUVA) for further application in different therapies.

Key words: Nanoparticles; benzopsoralen; vascular endothelial cell; PUVA; photochemistry

1. Introduction

The association of psoralens with ultraviolet A (UVA) irradiation (320–400 nm) is currently being employed in dermatology (orally or topically) (Ito, Aoshima et al., 2009; Makki, Muret et al., 1996; Pires, Honda et al., 2004; Stern, 2007; Weber, Schmuth et al., 2005). This combination is known as PUVA therapy (Canton, Caffieri et al., 2002; Gomes, Faustino et al., 2007; Lysenko, Melnikova et al., 2000; Machado, Miranda et al., 2001; Roop, Guy et al., 2004). This treatment is effective against diseases such as vitiligo, psoriasis, mycosis fungoides, and atopic eczema, among others (Adisen, Karaca et al., 2008; Creamer, Martyn-Simmons et al., 2007; Gambichler, 2009; Mariano, Vetrano et al., 2002; Saiad, Makki et al., 1997; Serrano-Perez, Gonzalez-Luque et al., 2008; Tatchen, Kleinschmidt et al., 2004; Tokura, 1999; Wackernagel, Hofer et al., 2006).

Most psoriasis drug development has focused on the T lymphocyte and its cytokines, which have been implicated in the changes seen in the skin of patients with psoriasis, including capillary dilation, epidermal ridge dilation, neutrophil proliferation, and plaque formation (Baier, Asadullah et al., 2006; Demidem, Taylor et al., 1991; Dupuy, Bagot et al., 1991; Giblin and Lemieux, 2006).

The physicochemical properties of drugs such as molecular weight, shape, charge and aqueous solubility determine the rate of diffusion through tissue (Minchinton and Tannock, 2006).

The photophysical properties of the benzopsoralen (BP) (3-ethoxycarbonyl-2H-benzofuro-[3,2-e]-1-benzopyran-2-one), have been recently investigated (Gomes, Lunardi et al., 2007a; Machado, Miranda et al., 2001; Oliveira, Raposo et al., 2003), and it has been shown that they can photochemically sensitize the generation of singlet oxygen with a quantum efficiency approaching unity (Machado, Miranda et al., 2001),

contrary to other compounds (8-methoxypsoralen, 5-methoxypsoralen, trimethylpsoralen) (Carter, McMacken et al., 1973; Chen and Lu, 1999; Collins, Wainwrigth et al., 1996; Legat, Wolf et al., 2001; Man, Dawe et al., 2004) usually employed in PUVA therapy. Unfortunately, most photosensitizing chemicals are also phototoxic to the skin, and skin contact with these molecules in the presence of UV irradiation results in sunburn, erythematic, and eventual edema (Middelkamp-Hup, Pathak et al., 2004; Roop, Guy et al., 2004), thus limiting the use of PUVA therapy for the treatment of skin disorders (Gomes, Faustino et al., 2006; Lindelöf, Sigurgeirsson et al., 1999; Middelkamp-Hup, Pathak et al., 2004).

A drug delivery system (DDS) may be used to enhance the action of PUVA therapy in special nanoparticles have been investigated for the delivery of different types of therapeutic agents including proteins, peptides, and DNA (Labhasetwar, Bonadio et al., 1999; Labhasetwar, Song et al., 1997). In addition to providing sustained release, nanoparticles can protect the encapsulated agent from enzymatic degradation (Alexis, 2005; Hedley, Curley et al., 1998; Yoshioka, Kawazoe et al., 2008). Since PLGA is a biodegradable and biocompatible polymer, it is well tolerated by the body and cells (Mooney, Sano et al., 1997; Wischke and Schwendeman, 2008; Yang, Li et al., 2009).

It is well known that endothelium is an important target for drugs because it is involved in a number of normal and pathophysiological conditions such as angiogenesis, atherosclerosis, tumor growth, myocardial infarction, limb and cardiac ischemia, restenosis, etc (Dass and Su, 2000; Davda and Labhasetwar, 2002; Martin and Murray, 2000; Parikh and Edelman, 2000). In skin disorders such as psoriasis there is now considerable evidence indicating that angiogenesis may, at least in part, play a role in increasing the psoriatic plaque (Hern, Stanton et al., 2005). Also the demonstration of

angiogenic activity in plaque skin has led some authors to investigate the possible benefits of antiangiogenic agents in the treatment of clinical lesions (Atherton, Wells et al., 1980; Dupont, Falardeau et al., 2002). However, the delivery of agents that induce destruction of the endothelial cells in skin vasculature, activated by light irradiation need to be investigated. Thus, the main objective of this work is to evaluate the morphological changes of vasculature in the rat skin after NP-BP with light irradiation. In this work we analyzed the rat skin and the mimetic vessel like a rat aorta to understand the uptake of the NP-BP by endothelial cell by using transmission electron microscopy and confocal microscopy.

2. Material and Methods

2.1. Chemicals

Poly(D,L-lactic-co-glycolic acid) (PLGA) 50:50, Mw 17 kDa (Sigma), polyvinyl alcohol (PVA) (13-23 kDa, 87-89% hydrolyzed) (Aldrich), and analytical grade dichloromethane (Merck) were used as supplied. The fluorescent bezopsoralen 3-ethoxy carbonyl-2H-benzofuro[3,2-e]-1-benzopiran-2-one (BP) was synthesized and supplied by Oliveira-Campos, from Minho University. All other chemicals were of analytical grade and were used without further purification.

2.2. Preparation of nanoparticles by the solvent evaporation technique

The NPs loaded or not with BP were prepared by solvent evaporation method (Gomes, Assuncao et al., 2007; Gomes, Lunardi et al., 2005). Typically, the organic phase consisted of 0.1 g of the PLGA 50:50 polymer and 10 mM of fluorescent BP dissolved in 10 mL of CH_2Cl_2. The dispersed phase was dropped into the homogeneous aqueous phase (50 mL of an aqueous phase containing 1% (w/v) of 88% hydrolyzed PVA as

dispersing agent) under ice cooling, using a high speed homogenizer (Ultra-Turrax T18-S18N-19G, IKA) for 60 s, at 14,000 rpm. Further solvent evaporation was carried out by gentle magnetic stirring at room temperature, for a period of 3–5 h. NP was recovered by centrifugation for 5 min, at 10,000 rpm and 4 ◦C. They were then washed (three times) with distilled water and lyophilized (Labconco®, USA). NP without BP was prepared by the same procedure. Dried NP was stored in a sealed glass vial and placed in a dessicator kept at 4 ◦C.

2.3. Particle size and Surface Charge (Zeta Potential)

Particle size and size distribution were determined by photon correlation spectroscopy (PCS) using the quasi-elastic light scattering technique, in a Zetasizer 3000 equipment (Malvern Instrument) equipped with a 10 mW He-Ne 633 nm laser beam, at 25^{o}C and at a scattering angle of 90°. For the particle size analysis, a dilute suspension (1.0 mg/mL) of NP was prepared in double distilled water and sonicated in an ice bath for 30 s. The zeta potential of the NPs in PBS buffer, (0.1mM, pH 7.4 1.0 mg/mL) was determined by using ZetaPlusTM in the zeta potential analysis mode.

2.4. Encapsulation efficiency

A weighed quantity of NPs was dissolved in methylene chloride. The BP content was assayed by spectrometry at 345 nm by using a calibration curve. The calibration curve for the quantification of BP was linear over the range of standard concentration between $1x10^{-7}$ a $1x10^{-3}$ $mol.L^{-1}$ with a correlation coefficient of R^2 =1.0. The encapsulation efficiency was calculated as following (Equation 1):

$$\text{Encapsulation efficiency } (\%) = \frac{\text{Amount of drug in microparticle}}{\text{Initial amount of drug}} \times 100 \qquad \text{(Equation 1)}$$

2.5. Nanoparticles Morphology: SEM Analysis

Scanning electron microscopy (SEM) was used to evaluate the shape of PLGA NPs. Samples were washed with sterile distilled water, fixed in 2.5% (v:v) glutaraldehyde (GA) in water, for 2 hours, washed again with water, dehydrated in a graded ethanol series and critical point dried. Samples containing NPs were mounted on aluminum stubs and due to their lack of electrical conductivity, coated with 50 nm gold coating under an argon atmosphere. NPs were examined and photographed using a EVO 50 (Zeiss) Scanning Electron Microscope operating at 20 kV in the traditional mode (SE1 detector).

2.6. Skin

Male Wistar rats weighing approximately 150 g (n= 4) were anaesthetized with a mixture of halothane, N_2O and O_2. After shaving and disinfection, 200 μL of a 25% suspension of BP-NP in 10 mM PBS, pH 7.4, was injected subcutaneously. At day 2 the rats were sacrificed and the NPs with surrounding tissue, skin, and underlying muscle were carefully dissected. The samples were fixed in either of the fixative agents already described, during the time shown in brackets: formalin 4% (24 h); or mixture of formalin (4%) plus glutaraldehyde (0.1%) in PBS (12 h); or glutaraldehyde (2.5%) in PBS (6 h). After fixation the samples were processed for microscopy analysis.

2.7. Transmission electron microscopy (TEM)

For the TEM studies, the samples were fixed in 2.5% glutaraldehyde in 0.1 $mol.L^{-1}$ sodium cacodylate buffer (pH 7.4) for 2h, at 25◦C. The fixed cells were then post fixed in 1% osmium tetroxide in the same buffer for 1 h, dehydrated in a graded acetone series, and embedded in epoxy resin. Ultrathin sections were contrasted with alcoholic

2% uranyl acetate and 5% lead citrate. Ultrastructural examination was performed with a transmission electron microscope Philips CM-100 (Philips Electron Optics, Eindhoven, The Netherlands).

2.8. Visualization of cellular uptake by confocal laser scanning microscopy

Male Wistar rats (400- 450 g) were killed by decapitation and all the procedures are in accordance with the Ethical Animal Committee of the University of São Paulo, Brazil. The thoracic aorta was quickly removed, cleaned of adherent connective tissues, and cut into rings (100 ± 20 μm thick). To image arterial cross sections, the aorta rings were placed vertically in a coverslip covered with poly-L-lysine. The tissue was loaded with fluorescent BP-NP by incubation in the normal Hanks solution 120 min in CO_2 incubator. After washing with Hanks solution, the segment was placed on a chamber (1.0 mL in volume). The chamber was placed on the stage of a confocal microscope from the bottom of the chamber through a water-immersion objective (63X). Excitation wavelengths and emission was set according to the fluorescence properties of the drug (Ex. 488 nm / Em. 532 nm)

2.9. Irradiation

BP-loaded nanoparticles (20 mg) were re-constituted with 2.0 mL of PBS pH 7.4 with 0.1% w/v Tween 80 in a glass vial which was shaken and briefly bath-sonicated. 1.5 mL of this suspension was then subcutaneously administered into the dorso lateral region of the neck. After 2 hours the region was irradiated using a 400-W mercury arc lamp was used as the irradiation source. A pass-band filter (Ocean Optics U360) filtered the radiation, ensuring the samples were irradiated with light of 360 ± 10 nm (maximum transmittance 69%). Typical irradiances of 0.030 to 0.035 W/cm^2 were used to deliver a

fluency of 1.0 J/cm^2. The irradiances were quantified using a PMA 2100 solar light radiometer. After light treatment, the cells were kept in the dark for 2 hr. Nonirradiated cells were kept in the dark for the same period of time. The ambient light was <0.001 mW/cm^2. Both irradiated and nonirradiated cells were processed by TEM.

2.10. Statistical analysis

Statistical analysis of the results was carried out using Student's t-test.

3. Results

3.1. Nanoparticles Morphology: SEM Analysis

The morphological analyses of the NPs were performed using scanning electron microscopy (SEM). Figure 1A shows a representative micrograph of a benzopsoralen-loaded nanoparticles, magnified at 20,000x. In all preparations reported in this paper the particles are spherical in shape, displaying a smooth surface. No meaningful difference was found between benzopsoralen loaded PLGA nanoparticles and the empty nanoparticles used as control. Figure 1B shows a laser scanning confocal microscopy of benzopsoralen loaded PLGA nanoparticles excited with 488nm.

FIGURE 1

3.2. Particle size and surface charge (zeta potential)

The distribution analyses (DLS) show a uniform size with varying diameters from 720 to 930 nm as presented in Figure 2 and confirmed by Figure 1.

FIGURE 2

The NPs colloidal stability was analyzed by measuring the NP zeta potential. In theory, more pronounced zeta potential values, either positive or negative, tend to stabilize particle suspension, since the electrostatic repulsion between particles with the same electric charge prevents aggregation of the nanoparticles. The particles consisting of PLGA-free dye were negatively charged (−3.2mV at pH 7.4) whereas the zeta potential measured for the NPs loaded with benzopsoralen was +17.3 ± 2.9 mV.

3.3. Encapsulation Efficiency

The drug entrapment efficiency parameter was calculated from the experimentally determined actual drug loading of the NP and the theoretical drug loading. The values obtained by this method 74±4% of incorporation.

3.4. Transmission electron microscopy (TEM) analysis

The histological evaluation of the vascular endothelial cells loaded with NP-PB with/without UVA irradiation was done by TEM. In Figure 3A it is presented the transversal cut of the control rat skin without the nanoparticle and irradiated with light at 360 ± 10 nm showing the intact vessel (black arrow-endothelial cell, star-myofibrils of muscle cell and bold arrow-muscle layer). In Figure 3B it is displayed the endothelial and muscle cells loaded with NP-BP without light irradiation. This image shows no alteration in the cytoplasm of the endothelial cell. In Figure 3C it is shown the damage in the cytoplasm of endothelial cell promoted by activation of NP-PB by irradiation (black arrow).

FIGURE 3

3.5. Laser Confocal Microscopy and image analysis

The uptake of NP-BP by endothelial cells was evaluated in aorta rings as a model incubated for 120 minutes and imaged with a confocal microscope. As shown in Figure 4A, phase contrast image of the aorta ring segment has undulation of the internal elastic membrane, which exists between vascular smooth muscle cells (VSMC-square) and endothelial cells (ECs-circle) layers. The endothelial layer with the uptake BP-NP (arrows) were imaged in xyz mode 1024x1024 pixel at 700Hz by a confocal scanning laser microscope (Leica TCS SP5). The BP-NP fluorescence was excited with the 488 nm line of an argon ion laser and the emitted fluorescence was measured at 490-550 nm as shown in Figure 4B. Serial z-sections of the cells, each 1 μm in thickness, demonstrated fluorescence activity in all the sections between 10 and 25 μm from the surface of the cells indicating that the nanoparticles were internalized by the cells and not simply bound to their surface (Figure not shown). Nanoparticles were mostly seen localized in the EC as showed in merged image (Figure 4C).

A major advantage of using the rat aorta artery segment was that SMCs and ECs were observed simultaneously at one confocal plane.

FIGURE 4

4. Discussion

The benzopsoralen was encapsulated in polymeric nanoparticles of PLGA using the solvent evaporation technique. The choice of loading conditions was dictated by the characteristics of both BP and the NPs matrices. The BP is a hydrophobic compound with low molecular weight which makes it able to be used with the PLGA polymer. This polymer is biocompatible and biodegradable and also has been approved by the

FDA for certain human clinical uses (Gomes, Lunardi et al., 2006a; Gomes, Lunardi et al., 2006b; Jain, 2000; Khang, Rhee et al., 2003; Sinha and Trehan, 2005; Thomas, Dean et al., 2006).

The morphological analysis of the nanoparticles was performed using SEM. In all preparations reported in this paper the particles are spherical in shape, displaying a smooth surface. No meaningful differences were found between the PLGA nanoparticles containing benzopsoralen (BP-NP) and the empty PLGA nanoparticles used as control in relation to external morphology. Similar behavior has been described in relation to external morphology using different drugs loaded into nano and microparticles when the solvent evaporation technique was applied (Gomes, Assuncao et al., 2007; Gomes, Faustino et al., 2007; Gomes, Faustino et al., 2006).

The success of this process depends primarily on the retention of the hydrophilic active compound within the polymer-containing organic phase from which the matrix is formed after evaporation of the solvent (Gomes, Barbougli et al., 2008; Gomes, Lunardi et al., 2007b). The parameter indicative of this behavior is the entrapment efficiency. The high value (74%) of the BP entrapment efficiency for the NP is consistent with the hydrophobic character of the compound.

According to the literature (Jeon, Jeong et al., 2000), the administration of particles with a diameter of several micrometers (larger than 6.0 μm) seems to be inefficient as a drug delivery system (DDS) because of its accumulation in lung capillaries and its difficult removal from the endothelial reticulum system (Gomes, Lunardi et al., 2006a; Jeon, Jeong et al., 2000).The particle size significantly affects the level of cellular and tissue uptake, and in some cell lines, only submicron size particles are efficiently taken up (Gomes, Faustino et al., 2006; Panyam and Labhasetwar, 2003). In this respect, the size of particles is important and should be characterized carefully.

The particles loaded with BP, evaluated by DLS, showed minimum dispersion size. Therefore, this method resulted in the preparation of particles, suitable for the use as a DDS.

The skin consists of five layers (deepest to most superficial): the stratum basale, the stratum spinosum, the stratum granulosum, the stratum lucidum, and the stratum corneum. It is also composed of four different types of cells: keratinocytes, melanocytes, Langerhan's, and Merkel cells (Peters, Weissman et al., 2000). Typically, it takes about four weeks for cells to move from the stratum basale toward the stratum corneum (Peters, Weissman et al., 2000). The keratinocytes in the basal layer divide once every 13 to 14 days (Greaves and Weinstein, 1995; Peters, Weissman et al., 2000). The surface cells slough off and are replaced by the underlying cells. In psoriatic skin, the cell cycle accelerates and the cells divide every 1.5 days, with maturation and shedding occurring in four days. Since the cells move to the surface so rapidly, they do not differentiate, thus forming scaly, inflamed, red skin (Greaves and Weinstein, 1995; Peters, Weissman et al., 2000).

Most psoriasis drug development has focused on the T lymphocyte and its cytokines, which have been implicated in the changes seen in the skin of patients with psoriasis, including capillary dilation, epidermal ridge dilation, neutrophil proliferation, and plaque formation. Transmission electron microscopy (TEM) demonstrates that the drug was entrapped in the NP and that it was able to successfully promote efficient photodamage in the target tissue and cells of PUVA therapy. The cellular uptake was also visualized by confocal microscopy. The BP shows a fluorescence emission at 532 nm and the NP-BP was uptaked by endothelial cells.

5. Conclusions

In this study, the hydrophobic compound (3-ethoxy carbonyl-2H-benzofuro[3,2-e]-1-benzopiran-2-one) was encapsulated in a biodegradable polymer matrix (PLGA) to generate stable nanoparticles. The present study demonstrates that the solvent evaporation method is a simple and suitable technique for the encapsulation of the hydrophobic compound, showing high encapsulation efficiency. The results displayed a good batch to batch reproducibility with respect to the particle characteristics (size and size distribution, encapsulation efficiency and release profile).

In summary, the results of this study suggest that the nanoparticle prepared with PLGA may provide a sustained drug-delivery system that can be used in order to obtain a local and systemic effect with no drug-related side-effect. Further studies are necessary to standardize the local tissue dose and the systemic prolonged exposure with maximal antineoplasic activity and minimal side-effect.

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