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Branched poly (lactic acid) microparticles for enhancing the 5-aminolevulinic acid phototoxicity

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ABSTRACT

An innovative microcarrier based on a carboxy-enriched and branched polylactic acid derivative was developed to enhance the in vitro phototoxicity of the photosensitizer and prodrug 5-aminolevulinic. Microparticles, prepared by double emulsion technique and loaded with the prodrug were carefully characterized and the effect of the polymer structure on the chemical, physical and biological properties of the final product was evaluated. Results showed that microparticles have a spherical shape and ability to allocate up to 30 μg of the photosensitizer per mg of carrier despite their difference in solubility. Release studies performed in various simulated physiological conditions demonstrate the influence of the branched structure and the presence of the additional carboxylic groups on the release rate and the possibility to modulate it. In vitro assays conducted on human epithelial adenocarcinoma cells proved the not cytotoxicity of the carriers in a wide range of concentrations. The hemocompatibility and surface proteins adsorption were evaluated at different microparticles concentrations to evaluate the safety and estimate the possible microparticles residential time in the bloodstream. The advantages, of loading 5-aminolevulinic acid in the prepared carrier has been deeply described in terms of enhanced phototoxicity, compared to the free 5-aminolevulinic acid formulation after irradiation with light at 635 nm. The obtained results demonstrate the advantages of the prepared derivative compared to the linear polylactide for future application in photodynamic therapy based on the photosensitizer 5-aminolevulinic acid.

1. Introduction

Photodynamic therapy (PDT) has been largely investigated for cancer treatment because of the non-toxic nature of the components as well as the minimal side effects which could improve the quality life of the patients [1–4]. PDT is based on the use of a photosensitizer agent and a particular type of light. It has received great attention for the treatment of different types of cancer due to the non-toxic nature of the components as well as the minimal side effects [4]. The photosensitizers (PS) alone are not harmful but are activated under irradiation at a specific wavelength. When exposed to the light, they produce a reactive singlet oxygen (ROS) which damage and cause the death of the nearby cells [5]. In addition, PDT appears to shrink or destroy the tumour mass by damaging the blood vessels preventing the cells to receive necessary nutrients, or by activating the immune system against the tumour cells. To be efficient, the PS should have a high absorption coefficient and quantum yield of triplet formation able to react with the target molecules [6]. The wavelength of the radiation used determines the penetration of the light into the body to reach and activate the PS [7–9]. Up to now, several PS have been developed either porphyrin or non-porphyrin based (Fig. 1). Porphyrins PS have a four pyrrole units (tetrapyrrole) linked by methin bonds. There are synthetics but also natural like pigments e.g. metallopigments heme, vitamin B12, chlorophyll and siroheme. Porphyrins and porphyrin-related dyes used in PDT have substituents in the peripheral positions of the pyrrole rings (1–8), on the four methine carbons and coordinated metals. These derivatives have been developed to optimize the water/lipid solubility, pK_a, stability, intracellular localization, tissue distribution and pharmacokinetics according to the needs.

Non-porphyrin based PS can be grouped in i) natural like psoralens, quinones, anthracyclins; ii) synthetic, xanthene, fluorescein, rhodamine and cyanine or iii) endogenously synthesized and degraded such as 5–ALA. 5-ALA and its derivatives received great attention in PDT and PDD (photodynamic diagnosis). They are prodrugs and need to be converted into protoporphyrin IX (PpIX), via the heme biosynthetic pathway in the mitochondria [10,11]. The application of 5-ALA in therapy is limited by its hydrophobic character which causes low cellular uptake and bioavailability and poor cell selectivity [12]. The most

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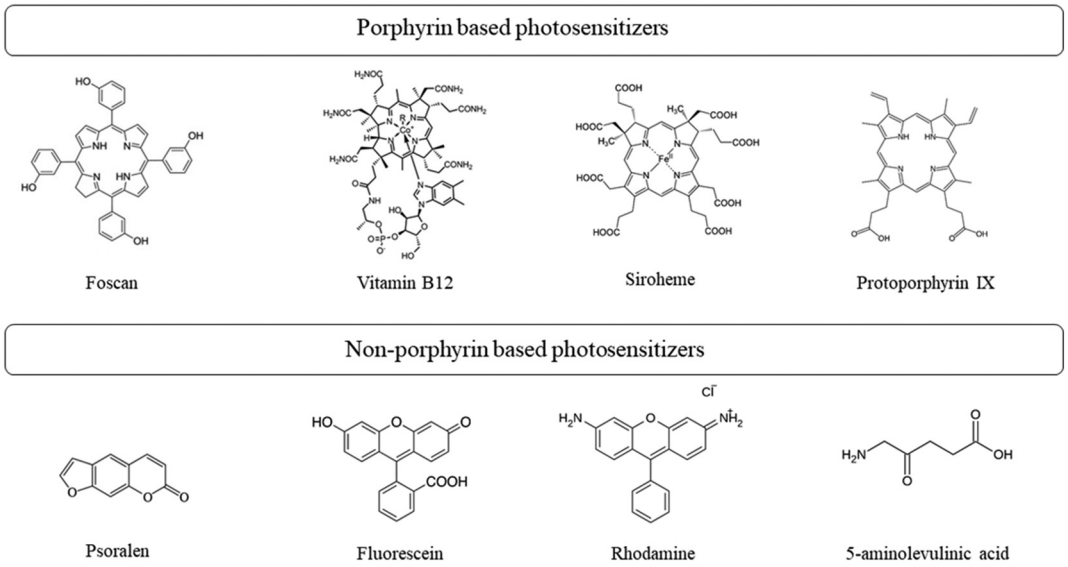

Fig. 1. Chemical structures of some representative porphyrin and non-porphyrin based photosensitizers.

innovative strategy to overcome these drawbacks, besides the chemical modification based on esterification (methyl, hexyl, lipophilic derivatives) [13], is represented by the used of specific carriers in nano and micro scale [14]. Polymer-based nano and macro particles demonstrate to have suitable properties to load various PS [15]. Is known that micro, as well as nanoparticles, are ideal vehicles for targeting solid tumors, because of their favourable bio-distribution, excellent bioavailability, the capability to protect the payload and release it at the target site in a predetermined way [16]. With particular regard to 5-ALA, the main advantages coming from the use of carrier are the improved permeability through the lipid barriers and the increase in the half-life, which stand for only 45 min in the free form.

One of the widely used polymer in the development of delivery vehicles is polylactic acid (PLA), due to chemical, physical properties, good biocompatibility and the possibility to control the biodegradation rate [17,18]. Up to know several PLA derivatives have been synthetized in order to obtain properties which are application-specific.

In this work, a PLA derivative with branched structure and enriched in carboxylic groups was synthetized with the aim to allocate 5-ALA in high concentration, release it in a controlled way and improve the in vitro phototoxicity to reduce the minimal dose required. The PLA derivative was prepared by polycondensation, the particles by double emulsion technique and characterized in terms of dimension, morphology, stability, release properties, hemocompatibility and protein adsorption. The advantages, of loading 5-aminolevulinic acid in the prepared carrier, in terms of phototoxicity after irradiation with 635 nm radiation were demonstrated using human epithelial adenocarcinoma cells.

2. Materials and Methods

2.1. Materials

L-Lactic acid (80% water solution), was purchased from Merci s.r.o., Czech Republic. Dimethyl sulphoxide in deuterated form (DMSO-d_6), pentetic acid (PA) (*N*,*N*-bis(2- bis(carboxymethyl)amino]ethyl)glycine, ≥ 99.5%), DL-lactic acid (90% water solution), hydrochloric acid (HCl, 30% for trace analysis), 5-aminolevulinic acid, MTT were supplied by Sigma Aldrich. The solvents acetone, methanol, indicator phenolphthalein, potassium hydroxide, (all analytical grade) were bought from IPL Lukes, Uhersky Brod, Czech Republic. Tetrahydrofuran (HPLC grade) was purchased from Chromservis, Czech Republic. All chemicals were used as obtained without further purification.

2.2. Synthesis and Characterization of Branched PLA

Carboxy-enriched branched PLA (BPLA) was synthetized following the procedure reported elsewhere [19]. Briefly, 1 g of PA was added to a two-neck round-bottom flask containing lactic acid water solution 80%. The mixture was heated at 110 o C for 1 h under reflux after that temperature was raised to 130 oC and reaction continued for 24 h at 1 kPa pressure. The obtained product was cooled at room temperature and then dissolved in acetone and subsequently precipitated into a mixture of methanol and distilled water at ratio 1:10 (*v*/v), filtered, washed with water and methanol and dried in vacuum oven at 30 °C for 48 h.

The molecular weight of the obtained product was determined by Gel Permeation Chromatography. The analysis was conducted on the HT-GPC 220 system (Agilent, UK). Samples were dissolved in THF (~3 mg·ml^{-1}) and filtered through a syringe filter (45 µm). Separation and detection took place on PL gel-mixed bed columns (1× MixedA, 300 × 7.8 mm, 15 µm particles +1× Mixed-B, 300 × 7.8 mm, 10 µm particles +1× Mixed-D, 300 × 7.8 mm, 5 µm particles) at 40 °C in THF. The flow rate equaled 1.0 ml·min-1 and injection volume was 100 µL. Weight average molar mass Mw, number average molar mass M_n, and molar-mass dispersity (Đ = M_w/M_n) were determined from the light scattering signal and the given results reflect absolute molecular weights. No calibration standards were applied, and dn/dc values were obtained through the mass constant for RI, assuming 100% mass elution from the columns.

^{1}H NMR measurements were performed on a Varian Unity Inova 400 spectrometer. The chemical shifts of signals in spectra were referenced to solvent peaks (^{1}H NMR (400 MHz, DMSO-d_6)): δ = 2.50 ppm. First order analysis was applied to evaluate the spectra. ^{1}H NMR spectra on the basis of the number for integrated signal areas (I(ppm)) given in subscript represent appropriate chemical shifts (I) that facilitate recognition of the characteristics described in the Table 2 reported in the supplementary material.

2.3. Preparation and Characterization of 5-ALA Loaded Microparticles

Bare and drug loaded microparticles were prepared by the double emulsion method, with minimal modifications, described elsewhere [20,21]. BPLA was dissolved in DCM (dichloromethane) and mixed with a buffer solution (pH 5) containing 5-ALA. The resulted mixture was sonicated for 5 min at 12000 rpm and then transferred into distilled water containing 1% polyvinyl alcohol, sonicated for a further minute

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