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Albumin-covered lipid nanocapsules exhibit enhanced uptake performance by breast-tumor cells

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ABSTRACT

Liquid lipid nanocapsules (LLN) represent a promising new generation of drug-delivery systems. They can carry hydrophobic drugs in their oily core, but the composition and structure of the surrounding protective shell determine their capacity to survive in the circulatory system and to achieve their goal: penetrate tumor cells. Here, we present a study of LLN covered by the protein human serum albumin (HSA) and loaded with curcumin as a hydrophobic model drug. A cross-linking procedure was performed to further strengthen the protective protein layer. Physicochemical properties and release kinetics of the nanocapsules were investigated, and cellular uptake and killing capacity were evaluated on the human breast-cancer line MCF-7. The nanocapsules exhibited a half maximal inhibitory concentration (IC_{50}) capacity similar to that of free curcumin, but avoiding problems associated with excipients, and displayed an outstanding uptake performance, entering cells massively in less than 1 min.

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1. Introduction

Controlled drug delivery may be considered a frontier area of pharmaceutical science and technology, involving a multidisciplinary scientific approach and contributing significantly to human health care. New delivery systems, compared to conventional ones, can improve efficacy and patient compliance and convenience. Due to the poor solubility of most anticancer drugs, current therapies must use solubilizer agents for intravenous administration, which provoke unpleasant secondary effects. Nanoparticle-based drug-delivery systems are suitable to overcome this solubility problem while offering other advantages, such as a high degree of biocompatibility and versatility [1], sustained delivery [2], and protection from chemical and physical degradation [3]. An important restriction for the use of nanoparticles (NP) in drug delivery is their potential toxicity [4]. A proposed solution for this limitation is to adopt, as nanocarriers, proteins that exit in the human body, such as albumin. Human serum albumin (HSA), adsorbed onto polystyrene nanoparticles, reportedly inhibits their phagocytosis and promotes prolonged circulation time in blood [5–7]. Therefore, HSA is considered a disopsonin, i.e. a molecule that can avoid clearance by the reticulo-endothelial system. On the other hand, albumin accumulates in malignant and inflamed tissues, and serves as the main nutrient for tumor growth [8]. For these properties, HSA has been proposed as an important tool to develop drug vehicles [9–11].

Lipid-based nanocarriers are being widely used for drug delivery, with some already passed for clinical use [12]. Examples for such lipid nanovehicles are solid lipid nanoparticles, nanostructured lipid carriers, and liquid lipid nanocapsules (LLN). LLN are colloidal systems consisting of an oily core covered by a protective polymeric shell. They can reach high drug-encapsulation efficiency due to their hydrophobic nature [13] and reduce tissue irritation at the deposition site due to their polymeric shell [14]. Surfactants and hydrophilic molecules such as chitosan, polyethylene glycol (PEG), poly(lactide-co-glycolide) (PLGA) or poloxamers, have been used to coat LLN [15].

Features such as the pharmacokinetics, biodistribution, and the colloidal stability of NP in biological media are governed largely by their surface properties. Most NP formulations are rapidly

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sequestered by cells of the mononuclear phagocyte system after intravenous administration. The choice of an appropriate coating polymer to decrease the interaction of the NP with serum components is important to achieve long blood-circulation time [16,17]. Recent studies have shown that the synthetic identity of NP plays an important role in the phagocytic cell uptake [18,19]. In addition, the physicochemical properties of the NP shell should influence the drug-release pattern. Human-serum albumin is a good candidate to be employed as external polymeric shell as it is a natural material with biocompatibility, biodegradability, and non-toxicity. This protein shell can be further strengthened by covalent cross-linking, and specific targeting molecules -that can recognize markers on the surface of cancer cells- may be attached by reaction with carbodiimide.

In a previous publication, we presented an extensive study concerning the influence of processing parameters on the properties of olive-oil nanocapsules stabilized by HSA [20]. The present study describes the development of nanocapsules with an olive-oil core covered by a cross-linked HSA shell. We have evaluated how the physico-chemical properties of these nanocapsules can influence their colloidal stability, drug release profile, and cellular uptake. For a hydrophobic drug model, we have chosen curcumin, a natural product with low intrinsic toxicity but recognized medicinal properties such as anti-oxidant [21], anti-inflammatory [22], anti-Alzheimer's disease [23], and anti-tumoral [24] activity.

2. Materials and methods

2.1. Reagents

Curcumin, coumarin-6, human serum albumin, olive oil, oleic acid, glutaraldehyde (GAD), poloxamer F-127, MTT assay reactive [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI), dimethyl sulphoxide (DMSO), L-glutamine, sodium bicarbonate, Hepes buffer, Nile Red, and penicillin/streptomycin solution were purchased from Sigma-Aldrich (Madrid, Spain). DMEM (Dulbecco's modified Eagle medium), MEM (minimum essential medium), and FBS (heat-inactivated fetal bovine serum) were purchased from Thermo Fisher Scientific (Gibco, Grand Island, NY, USA). All aqueous solutions were prepared using ultrapure water from a Millipore Milli-Q Academic pure-water system.

2.2. Preparation of LLN

LLN were prepared using a solvent-displacement method as previously described [20]. Briefly, as a general working scheme, a solution containing 300 μl of olive oil, 37.5 ml of ethanol and different amounts of curcumin was poured into 40 ml of an aqueous phase containing HSA to form an emulsion. The dispersion became turbid immediately because of the formation of nanocapsules. After 10 min of stirring, 250 μl of a 0.16% solution of GAD was added to the dispersion to cross-link the HSA coating molecules. The mixture was left under continuous stirring over a time period of 15 min at 25 °C. Subsequently, the ethanol was evaporated under vacuum at a temperature of 34 °C in a rotary evaporator and the dispersion thoroughly dialyzed against and low-ionic-strength phosphate buffer at pH 7. Similar LLN were prepared without curcumin to be used as blanks, as well as without the GAD cross-linking process for comparison purposes.

The amount of encapsulated curcumin in the nanocarriers was determined by UV–vis spectrophotometry after their disaggregation with propanol. Upon centrifugation at 14,000 rpm for 10 min, the supernatant was collected and the amount of curcumin determined by absorption at 430 nm using a UV–vis spectrophotometer (BioSpectronic Kinetic Spectrophotometer, Eppendorf, Germany). The curcumin concentration was calculated by appropriate calibration curve of free curcumin in propanol ($R^2 > 0.999$). Each batch sample was measured in triplicate.

LLN loaded with the fluorophore coumarin 6 were also prepared to compare the uptake efficiency of nanocapsules with different shells (protein, poloxamer, and a mixture of poloxamer and oleic acid). The preparation method in all cases was the same as previously described, changing curcumin to 0.1 mg of coumarin 6 in the three samples. HSA was replaced by 200 mg of Poloxamer F127 in the PL-C sample, and by 100 mg of Poloxamer F127 and 125 mg of oleic acid in the PLOA-C sample.

2.3. Morphology of nanocapsules

The aspect of LLN was assessed by Cryo-Transmission Electron Microscopy (Cryo-TEM) technique. Five microliters of sample were placed on a glow-discharged holey-type carbon-coated grid (Quantifoil R2/2), and vitrified in liquid ethane using a Vitrobot Mark IV (FEI), under controlled conditions. Samples were maintained at a temperature of approximately −170 °C in liquid nitrogen until they were transferred into a cryo-holder (Gatan), using a cryo-transfer stage. Imaging was carried out using FEI Tecnai G2 20 TWIN transmission electron microscope operating at 120 kV and at a nominal magnification of 100,000–150,000× under low-dose conditions. Images were recorded with a 2k × 2k MP side-mounted CCD camera (Olympus Veleta). Measurements were performed in the Andalusian Centre for Nanomedicine and Biotechnology, BIONAND (Málaga, Spain).

2.4. Measurement of size and surface potential of LLN

The particle-size distribution and zeta potential of LLN were determined by dynamic light scattering (DLS) using a Zetasizer Nano-S system (Malvern Instruments, UK). The self-optimization routine in the Zetasizer software was used for all measurements, and the zeta-potential calculated according to the Smoluchowsky theory. After a 100-fold dilution with a low ionic strength (2 mM) phosphate buffer at pH 7, measurements were performed at 25 °C in triplicate.

2.5. Determination of drug-release kinetics

The in vitro release kinetics of the curcumin-loaded LLN was undertaken by a dialysis method. For this, 2 ml of nanocapsule suspension was stuffed into a dialysis sac and placed in a 200-ml flask containing phosphate buffer at pH 7.4 in a water bath at 37 °C under magnetic stirring at 300 rev/min. At predetermined time intervals, 50 μl aliquots were extracted from the LLN suspension. The remaining amount of curcumin loaded in the nanocapsules was determined as previously described.

2.6. Cell line and culture conditions

The human breast-cancer MCF-7 cell line (American Type Culture Collection, ATCC) was grown at 37 °C in an atmosphere containing 5% CO_2 with DMEM supplemented with 10% (v/v) FBS, 2% L-glutamine, 2.7% sodium bicarbonate, 1% Hepes buffer, and 1% penicillin/streptomycin solution.

2.7. Uptake studies

With the aim of comparing the effectiveness entering cells of different nanocapsule shells, an initial study was performed with coumarin 6-labeled nanocapsules. For this, MCF-7 (5×10^3) cells were seeded into 6-well plates under the culture conditions

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