European Journal of Pharmaceutics and Biopharmaceutics 127 (2018) 432–442

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

An insight of *in vitro* transport of PEGylated non-ionic surfactant vesicles (NSVs) across the intestinal polarized enterocyte monolayers

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ARTICLE INFO

Keywords:
PEGylated non-ionic surfactant vesicles
Transepithelial electrical resistance analysis
In vitro biological barrier transport
Caco-2-cell monolayers
Nanoparticle tracking analysis

ABSTRACT

PEGylated non-ionic surfactant-based vesicles (NSVs) are promising drug delivery systems for the local, oral and systemic administrations of therapeutics. The aim of this study was to test the cellular biocompatibility and transport of Nile Red-loaded NSVs (NR-NSVs) across the Caco-2-cell monolayers, which represent an *in vitro* model of human intestinal epithelium. The NR-NSVs assumed a spherical shape with a mean size of 140 nm, and a narrow size distribution. The NR-NSVs did not modify Caco-2 cell viability, which remained unaltered *in vitro* up to a concentration of 1 mM. The transport studies demonstrated that the NR-NSVs moved across the Caco-2 monolayers without affecting the transepithelial electrical resistance. These results were supported by flow cytometry analysis, which demonstrated that NR-NSVs were internalized inside the Caco-2 cells. Nanoparticle tracking and Transmission Electron Microscopy (TEM) analysis showed the presence of NR-NSVs in the basolateral side of the Caco-2 monolayers. TEM images also showed that NSVs were transported intact across the Caco-2 monolayers, thus demonstrating a predominant transcytosis mechanism of transport through endocytosis. The NSVs did not affect the integrity of the membrane barrier *in vitro*, and can potentially be used in clinics to increase the oral bioavailability and delivery of therapeutics.

1. Introduction

Oral drug administration is widely used in clinics to treat many diseases due to the fact that it is painless and that patients can medicate themselves which allows it to meet with a great degree of patient compliance and this, in turn, means a good degree of adherence to therapeutic regimens. Oral therapy is also cheaper than systemic therapy in the treatment of chronic diseases, and its regime is flexible and easy to manage as compared with systemic administration. Unfortunately, the physiological barriers (gastric acid pH), the poor water solubility and stability of drugs, and their biopharmaceutical features (retention, permeation and metabolism) limit the oral absorption of both hydrophilic and hydrophobic drugs [1,2]. Even though the benefits for patients are greater than the drawbacks, oral administration represents the main therapeutic option for treating chronic diseases. Physiological hurdles do exist though, and cannot be overcome using conventional medicines. In this attempt, colloidal nanoparticles were developed to deliver payloads, and modulate and target drugs to specific tissues after oral administration [3]. Specific polymeric nanoparticles, micelles and liposomes were designed to promote oral absorption, improve the bioavailability of drugs, and allow their passage through the tight junctions (TJs). These colloidal nanoparticles can resist inactivation by acid (pH 2–4) and digestive enzymes, and protect payloads from degradation following oral administration, thus modulating drug release and targeting specific tissues [4–8]. The mucus of the gastro-intestinal (GI) tract can also modify the oral delivery of colloidal nanoparticles besides their transport across the GI barrier, thus affecting the therapeutic response of the drug in various acute and chronic diseases [9–11]. Size, surface properties, and particle concentrations can all influence the transport of colloidal nanoparticles

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https://doi.org/10.1016/j.ejpb.2018.03.013
Received 21 December 2017; Received in revised form 16 March 2018; Accepted 28 March 2018
Available online 29 March 2018
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across the human mucus monolayer [9,12].

In fact, the experimental modifications of the surfaces of the colloidal nanoparticles allowed them to overcome the mucus barrier, and increased their penetration across the epithelial cells of the GI tract [3]. Polyethylene glycol (PEG), a food and drug administration (FDA)-approved hydrophilic polymer, is currently being used to overcome the monolayer mucus barrier of the GI tract due to its long hydrophilic, polyethylene-oxide chains, which decrease the uptake of nanoparticles mediated by mononuclear phagocytic system (MPS), avoid interaction between nanoparticles, and stabilize the nanoparticles in biological fluids [13,14]. Previous studies demonstrated that PEG-coated liposomes, orally administered to deliver insulin in diabetic rats, significantly increased their anti-diabetic response to insulin, and provided a sustained hypoglycemic effect in hyperglycemic rats as compared to insulin delivered using naked liposomes. The resulting data demonstrated that the PEGylated liposomes were stable at the acid pH of the GI tract, prevented the degradation of bile salts, and interacted with the intestinal wall by adhering to the mucous layer [15]. Moreover , PEGylation facilitated the diffusion of the nanoparticles across the mucus barrier due to the effect of the interpenetration of the polymer network between the PEG chains and the mucus mesh fibers, and/or the hydrogen bonding that comes about between the ether oxygen atoms of PEG and the polysaccharide residues of glycosylated mucins [16]. The physicochemical properties of PEG such as density, molecular weight, and pending chain derivatives, can affect the diffusion of PEGylated nanoparticles through human mucus. In particular, several studies have demonstrated that the increase of PEG density and the decrease of the molecular weight of PEG increased the penetration of the nanoparticles in highly viscoelastic human mucus [17–20].

Through PEGylation, researchers can develop colloidal nanoparticles which can effectively penetrate the mucus layer and then accumulate on the surfaces of epithelial cells. Nanoparticles can move across the intestinal epithelium, and reach the blood stream by way of two different routes: (i) paracellular, and (ii) transcellular pathways. Paracellular transport involves the passive diffusion of drugs through the intercellular spaces between adjacent epithelial cells; however this is limited by the presence of the TJs [21,22]. The TJs provide a crucial structural support to the intestinal epithelium, maintain the constitutive cells in a cohesive and polarized condition and play a pivotal role in the diffusion of drugs across the intestinal monolayer [23]. The TJs, being the closely associated areas between two cells forming a virtual barrier that is impermeable to fluid, have pore sizes of 10–30 Å, and play an important role in controlling the permeability of epithelial cells. TJs also control the permeation of small hydrophilic drugs across the epithelium of the GI tract [1].

Transcellular transport includes passive diffusion, carrier-mediated transport, endocytosis, and transcytosis [24]. Passive diffusion depends on the size of nanoparticles, which limits their diffusion through cellular membranes and barriers. In order to overcome these drawbacks, the colloidal nanoparticles penetrate the cells by endocytosis [1,25]. The endocytic pathways include: (i) clathrin-mediated endocytosis, (ii) caveolae-mediated endocytosis, (iii) phagocytosis, (iv) macropinocytosis, (v) clathrin and caveolae-independent endocytosis [26,27]. In addition to using endocytic pathways, colloidal particles can also pass across the epithelial cell through transcytosis-mediated transport, which includes both endocytosis and exocytosis. The transcytosis of nanoparticles through epithelial cells involves cell-surface binding, endocytosis at the apical side of cell membranes, intracellular trafficking, and release at the basolateral side (exocytosis) of the cell membrane [1,28,29].

In a previous work, we demonstrated that NSVs of polysorbate 20, which contain repeated polyethylene oxide units similar to the PEG in the hydrophilic region of the surfactant, were stable and mucoadhesive *in vitro* in simulated GI fluids [30]. NSVs are stable, safe, biocompatible, cheaper than liposomes, easily stored, and increased the therapeutic efficacy of drugs *in vitro* and *in vivo* [31–33].

In this study, we investigated the physicochemical properties, such as average sizes, size distribution, zeta-potential, and morphology of the NSVs, their cytotoxicity (Caco-2 cells) and intracellular uptake, besides the transport of PEGylated NSVs through the Caco-2 cell epithelium barrier model. Caco-2 cells are basically used as *in vitro* models of the intestinal barrier and are approved by the FDA as a model of the human intestinal barrier. They are useful for studying the transport of drugs through the GI tract [34]. However, the intestinal epithelium consists of different types of cells, such as enterocytes, mucus-producing goblet cells, endocrine cells, and M cells. The Enterocytes make up the main population of GI cells, while goblet cells account for 10–24% [35]. Co-cultures of Caco-2 and goblet cells, i.e. HT29-MTX and HT29-H, are used to develop *in vitro* models of the human intestinal barrier that secretes mucus [35,36]. In order to mimic the intestinal barrier of the human GI tract, this barrier needs for the ratio between the Caco-2 and goblet cells to be optimized. In addition, there may be basic differences between the composition of the mediums and the cell culture times of the Caco-2 and goblet cells. In this attempt, we used only Caco-2 cells with the aim of obtaining a polarized epithelium model similar to that of the GI tract so that the interaction between the epithelium and PEGylated NSVs could be studied.

2. Materials and methods

2.1. Materials

Tween® 20 (Tw20), sephadex G-75, 9-(diethylamino)-5H-benzo[R] fenossazin-5H-one (Nile-Red or NR) were obtained from Acros Organics (Acros Organics BVBA, Geel, Belgium). Human epithelial colorectal adenocarcinoma cells (Caco-2 cells), trypan blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), Nonidet P-40, cholesterol (CHOL) and ribonuclease (RNase) A, chlorpromazine, methyl-β-cyclodextrin and genistein were purchased from Sigma-Aldrich (Sigma Aldrich SRL, Milan, Italy). Fetal Bovine Serum (FBS), trypsin-EDTA solution 1×, Phosphate Buffer Saline (PBS), Dulbecco's Modified Eagle Medium (DMEM), glutamine, penicillin and streptomycin were purchased from Euro Clone (West York, UK). 12-BD Falcon™ cell culture inserts (pore size 0.4 μm, growth area 0.9 cm^2) were obtained by Becton Dickinson (Milan, Italy). All other products and reagents were of analytical grade.

2.2. Synthesis and purification of NSVs

PEGylated non-ionic surfactant vesicles (NSVs) were made from Tw20 and CHOL at the same molar concentration (15 mM:15 mM). NSVs were obtained using the thin-layer evaporation method as previously reported [30]. The resulting film was then hydrated by adding the HEPES buffer (10 mM, pH = 7.4). NR-NVs were obtained by adding Nile Red (10 μM) to the Tw20/CHOL organic mixture before forming the surfactant film. The resulting NSVs were vortex-mixed for 5 min and then sonicated for 10 min at 60 °C using a probe sonicator (Hielscher, model UP200H, Teltow, Germany) equipped with an exponential microprobe operating at 24 kHz and amplitude of 60%, in order to obtain unilamellar suspensions. Both empty and fluorescent NSVs were then purified through Sephadex G-75 glass columns to remove the excess of un-assembled surfactants, fluorescent probe and cholesterol.

2.3. NSV characterization

2.3.1. Size and zeta potential measurements

The average size and size distribution of NSVs were measured using a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). Briefly, samples were diluted 1:50 (v/v) in HEPES buffer (10 mM, pH = 7.4) to avoid multiscattering phenomena. The average size and size distribution were measured using photon correlation spectroscopy in line with cumulant mode fitting (Malvern Instruments Ltd.,

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