Colloids and Surfaces B: Biointerfaces 169 (2018) 444–452

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Membrane permeation of giant unilamellar vesicles and corneal epithelial cells with lipophilic vitamin nanoemulsions

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ARTICLE INFO

Keywords:
Nanoemulsion
Lipophilic vitamin
Giant unilamellar vesicle
Morphological change
Corneal epithelial cell
Membrane permeation

ABSTRACT

Nanoemulsions of a lipophilic vitamin, retinol palmitate (vitamin A; VA), have a therapeutic effect on corneal damage. The nanoemulsion based on a triblock-type polymer surfactant with polyoxyethylene and polypropylene, $EO_{100}PO_{70}EO_{100}$ (EOPO) showed superior efficacy, as compared with a nanoemulsion based on polyoxyethylene (60) hydrogenated castor oil (HCO). We studied the mechanism of VA nanoemulsions related to efficacy from the viewpoint of the interaction with plasma membrane–mimicking giant unilamellar vesicles (GUVs) and the plasma membrane permeation in corneal epithelial cells. When nanoemulsions and GUVs doped with fluorescent compounds were mixed each other, and observed by confocal laser microscopy, EOPO nanoemulsions induced endocytic morphological changes like strings and vesicles of the bilayer drawn inside a GUV by budding. Judging by isothermal titration calorimetry and ζ potential measurements, the EOPO nanoemulsions seemed to have stronger hydrophobic interactions with the lipid bilayer because of lower coverage of the core interface. Next, when the nanoemulsions prepared with a pyrene derivative of retinol (VApyr) were applied to corneal epithelial cells, the EOPO nanoemulsions greatly permeated the cells and gathered around the cell nucleus, as compared with HCO nanoemulsions. Furthermore, according to the three-dimensional images of the cell, it was found that the vesicles that absorbed nanoemulsions formed from the plasma membrane as real endocytosis, and were transported to the area around the nucleus. Consequently, it is likely that EOPO nanoemulsions entered the cell by membrane-mediated transport, delivering VA to the cell nucleus effectively and enhancing the effects of VA.

1. Introduction

The oil-in-water (O/W) type of nanoemulsion, where lipophilic active ingredients can be dispersed in an aqueous medium (and this state can remain for a long period), has been widely applied in the fields of pharmaceutical and cosmetics manufacturing and food production [1,2]. Because the size and interface state of a nanoemulsion can be changed by altering the composition and preparation method, controlling the structure necessary for a carrier of active ingredients has been extensively studied [3–5]. Particularly, a nanoemulsion is important for improving the bioavailability of active ingredients [6–8] because particles with a diameter of 100 nm or less can easily enter the cell by endocytosis through membrane movements [9,10].

On the other hand, retinol palmitate (vitamin A; VA), a lipophilic vitamin, is known to have beneficial effects on the maintenance of a good condition of eyes [11] and on the function of the skin and mucosa [12,13]. In particular, when a VA nanoemulsion is used as eyedrops, it was found that the VA nanoemulsion promotes hyaluronic acid and mucin production in corneal epithelial cells and has an effective therapeutic action on a damaged cornea [14–16]. When polyoxyethylene (60) hydrogenated castor oil (HCO) and a triblock-type polymer surfactant of polyoxyethylene and polypropylene, $EO_{100}PO_{70}EO_{100}$, (EOPO) were tested as emulsifiers for VA, the EOPO nanoemulsion was superior to the HCO nanoemulsion in terms of the damage alleviation effect in dry eyes in a small-scale clinical trial [17]. It is thought that those nanoemulsions have different mechanisms of membrane permeation or of the delivery to the cell nucleus because the production of hyaluronic acid and mucin starts in the nucleus. Generally speaking, it is known that after VA is ingested, VA associates with retinol-binding protein (RBP) via the liver and is transported from blood to the cell interior through the RBP transporter in the plasma membrane [18–21]. In the case of direct application of eyedrops to the cornea, however, VA

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https://doi.org/10.1016/j.colsurfb.2018.05.052
Received 26 December 2017; Received in revised form 8 May 2018; Accepted 23 May 2018
Available online 24 May 2018

molecules in the nanoemulsion cannot bind to RBP and cannot be internalized via this transporter. It is believed that another membrane permeation route of a nanoemulsion is involved.

In a nanoemulsion, there are emulsion droplets that are oil droplets adsorbing a surfactant and free surfactant molecules that are not adsorbed on oil droplets. It is expected that both emulsion droplets and free surfactant molecules interact with the plasma membrane and contribute to membrane permeation. Thus far, the interaction with the plasma membrane has been studied in terms of dispersion, solubilization, molecular dynamics (by means of multi- or unilamellar vesicles) and regarding internal pressure (by means of a lipid monolayer) [22–24].

In general, it is known that the lipid bilayer consisting of unsaturated lipid, saturated lipid, and cholesterol separates partitions into a liquid disordered (L_d) phase and a liquid ordered (L_o) phase, which are rich in unsaturated lipids and in saturated lipids and cholesterol, respectively, at lower temperatures [25,26]. Actually, it is proposed that the raft domains that are enriched in saturated lipids and cholesterol may be formed in real cells [27,28]. The phase separation in the lipid bilayers has attracted our attention because of the relations with the raft model. Therefore, the effects of the lipid types [29,30], additives [31,32], tension of the membrane [33–35], hydrostatic pressure [36], and of other factors on the phase separation have been discussed. Because the lipid bilayers can be transformed easily by external stimuli, the dynamic changes of the membrane morphology caused by osmotic pressure [37] and an electric field [38] have been reported. Moreover, there are many reports about the link between the phase separation and membrane transformation related e.g. to the budding of domains [39,40], osmotic pressure [41,42], and a bilayer containing charged lipids [43]. It is widely accepted that addition of a surfactant leads to a membrane transformation. Discussions about the links between the phase separation and the transformation by a surfactant have also emerged [42–44].

Nonetheless, the influence of nanoemulsions, in which emulsion droplets and a free surfactant coexist, on giant unilamellar vesicles (GUVs) has not been investigated yet. Furthermore, there are few reports addressing the relations between the morphological changes in the GUV and the permeation of the plasma membrane by nanoemulsions. The aims of this work were to determine possible differences in membrane interactions between HCO and EOPO nanoemulsions by examining the morphological changes in plasma membrane–mimicking GUVs, and to discuss the drug delivery function of VA nanoemulsions related to the therapeutic effect on the damaged corneal cells by confirming the mechanisms underlying permeation of the plasma membrane of corneal epithelial cells.

2. Materials and methods

2.1. Materials

HCO and EOPO were purchased from Nikko Chemicals Co., Ltd., and BASF Japan, Ltd., respectively. Their molecular structures were showed in Scheme 1. Retinol palmitate (VA) was purchased from DSM Nutrition Japan Co., Ltd., and *dl*-α-tocopherol (Vitamin E: VE) from Rikenvitamine Co., Ltd.

1-Pyrenebutyric acid, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI HCl), triethylamine (Et_3N), 4-dimethylaminopyridine (DMAP), potassium carbonate (K_2CO_3), and 1,2-dichloroethane (CH_2Cl_2), which were used to synthesize the pyrene derivative of VA, were purchased from TOKYO Chemical Industry Co., Ltd. 1,2-Dioleoyl-*sn-glycero*-3-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn-glycero*-3-phosphocholine (DPPC), and cholesterol were purchased from Avanti Polar Lipids, Inc., and bovine brain ganglioside ammonium salt (GM1) from Calbiochem (Merck). The lipophilic fluorescent compound perylene was purchased from TOKYO Chemical Industry Co., Ltd., and the fluorescent lipid N-(rhodamine red-X)-1,2-dihexadecanoyl-*sn-glycero*-3-phosphoethanolamine triethylammonium salt (Rho-DHPE) and Alexa Fluor 555–conjugated cholera toxin subunit B (CTB-555) from Invitrogen (Thermo Fisher Scientific Inc.). Human Corneal Epithelial Cells 2 (HCEC-2) and the dedicated growth medium were purchased from Kurabo Industries, Ltd.

Ultrapure water (specific resistance ≥18 MΩ) was obtained by means of a Millipore Milli-Q purification system.

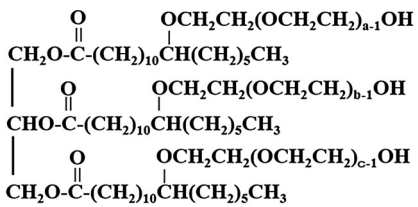
2.2. Methods

2.2.1. Synthesis of fluorescently labeled VA

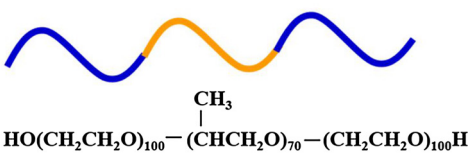
The fluorescent labeling of VA was performed by the following procedure (The synthetic route was shown in Scheme 2 in the supporting materials). VA (2.05 g, 3.8 mM) was dissolved in 20 mL MeOH and hydrolyzed to retinol by stirring for 2 h with K_2CO_3 (0.635 g, 4.6 mM) and a little CH_2Cl_2 at room temperature (RT). The retinol was isolated by silica gel column chromatography using the mixed solvent hexane:ethyl acetate = 5:1(v/v). Pyrenebutyric acid (0.532 mg, 1.86 mM), EDCI HCl (0.431 mg, 2.23 mM), Et_3N(160 μL, 2.23 mM), and DMAP (97.2 mg, 0.4 mM) were added to the retinol (0.9 g, 3.1 mM) in 10 mL CH_2Cl_2, and the mixture was stirred for 5 h at RT. After the reacted solution was washed with water and brine, pyrene-labeled retinol (VApyr) was isolated and purified by silica gel column chromatography using the mixed solvent hexane:ethyl acetate = 8:1. The chemical structure of VApyr was determined by nuclear magnetic resonance (NMR) and infrared (IR) spectroscopy. It was confirmed by thin-layer chromatography (TLC) that there were no byproducts.

2.2.2. Preparation and characterization of nanoemulsions

The VA nanoemulsions were prepared by the agent-in-oil method. The prescribed amounts of VA (or VApyr), VE, and surfactant were added into a light-shielding vial, and mixed at 70 °C until homogeneity. The mixture was added drop-wise to water at 80 °C with stirring until the solution became clear. The final concentration of VA and VE were 0.0285 and 0.05 g/mL, respectively. That of surfactants as emulsifiers were 0.1% and 0.5% HCO for HCO nanoemulsion S and Q, and 0.6% and 1.0% EOPO for EOPO nanoemulsion X and Y, respectively. (Their compositions are showed in Table S1 in supporting materials. These



POE(60)hydrogenated caster oil (HCO)
(a + b + c = 60)



Triblock type polymer surfactant
$EO_{100}PO_{70}EO_{100}$ (EOPO)

Scheme 1. Molecular structure of emulsifiers.