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# Protective effect of quercetin and rutin encapsulated liposomes on induced oxidative stress

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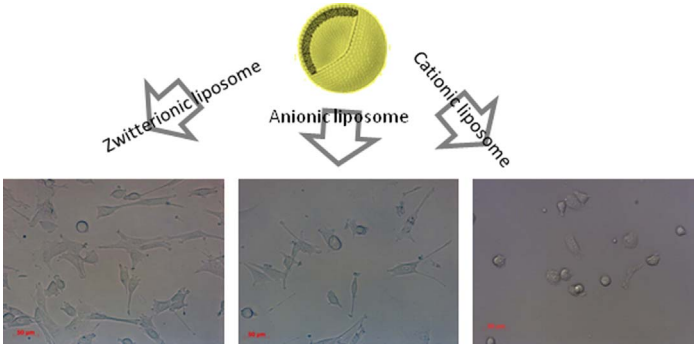
## HIGHLIGHTS

- Biological response to different surface liposome charge
- Better cell internalization of antioxidant molecules
- Cell protection from oxidative stress processes

## GRAPHICAL ABSTRACT

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## ABSTRACT

Natural antioxidants show many pharmacological properties, but poor solubility and inability to cross cell membrane. Liposomes are biocompatible and phospholipid vesicles able to carry hydrophilic, hydrophobic, and amphiphilic molecules. This paper focus on the synthesis of anionic, cationic and zwitterionic liposomes, loaded with quercetin or rutin, and on the evaluation of their cytotoxicity and protective effects against oxidative stress.

Chemical characterization was obtained by dynamic light scattering and z-potential experiments. In vitro cell behavior was evaluated by Neutral Red Uptake test.

All liposomes, empty and loaded with antioxidants, are stable. The cytotoxicity of both quercetin and rutin encapsulated in zwitterionic and anionic liposomes is higher than that of their solutions. Quercetin and rutin loaded in cationic liposomes are able to inhibit the toxic effect of empty liposomes. The encapsulation of rutin at $5.0 \times 10^{-5}$ and $5.0 \times 10^{-4}$ M, in zwitterionic and anionic liposomes, protects fibroblasts by $H_2O_2$ treatment, while the loading with quercetin does not have effect on improving cell viability.

All data suggest that the tested liposomes are stable and able to include quercetin and rutin. The liposomes encapsulation of antioxidants makes easier their internalization by cells. Moreover, zwitterionic and anionic liposomes loaded with rutin protect cells by oxidative stress. Liposomes stability together with their good in vitro cytocompatibility, both empty and loaded with antioxidant molecules, makes these systems suitable candidates as drug delivery systems. Moreover, the encapsulation of rutin, is able to protect cells by oxidative stress.

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## 1. Introduction

Antioxidants are biomolecules having the ability to protect organisms from diseases associated with oxidative stress, including cardiovascular diseases, cancer, inflammation, and other degenerative disorders [1]. Flavonoids, a group of naturally occurring benzo-γ-pyrone derivatives, have a large number of biological effects and particularly strong antioxidants and free radical scavengers [2,3]. Flavonoids may help to prevent and/or reduce oxidative-inflammatory status related to obesity through the regulation of different molecular pathways, such as mitogen-activated protein kinase (MAPK), adenosine monophosphate-activated protein kinase (AMPK), peroxisome proliferator-activated receptor-α and sterol regulatory element-binding protein-1c pathways [4].

Rutin (2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-[[(2*R*,3*R*,4*R*,5*R*,6*S*)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxymethyl]oxan-2-yl]oxychromen-4-one, R), is a flavonol glycoside, that shows various pharmaceutical proprieties, like anti-inflammatory, anti-hypertensive, anti-hemorrhagic, that have been attributed to its ability as free radicals scavenger [5]. It has been reported that such activity of polyphenols is highly sensitive to the chemical properties like solvent polarity, micellar solution, etc. [6].

Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one, Q) is a lipophilic compound, of great interest for human nutrition and functional foods [7,8] and it is the most abundant flavonoid in human dietary sources [9]. The anti-obesity effect of quercetin is mediated by the AMPK and MAPK signaling pathways [10,11]. Apart from its antioxidant capacity quercetin is also associated with anti-viral, anti-inflammatory, anti-bacterial and muscle-relaxing properties [12].

Jan et al. (2012) also reports substantial evidence related to the chemo-preventive properties of quercetin against certain types of cancers (including bladder, prostate, esophagus and stomach), as oxidative free radicals are considered to be one of the main causes of cancers formation, as well as tumor proliferation [12]. Many studies have demonstrated that levels of low molecular weight compounds in foods, such as beneficial secondary metabolites, can be affected by heat treatments that food undergo prior to consumption. Moreover, post-harvest processing can seriously reduce the level of phytochemicals in plant materials, by as much as 70% in some instances [13].

Quercetin and rutin protect against ultraviolet radiation damage, and both has been reported to have many biological activities such as the reduction of age-associated cell damage induced by cellular production of Reactive Oxygen Species (ROS) [14,15].

Due to their poor solubility in hydrophilic solutions (rutin: water solubility 0.125 mg/dm$^3$, 0.2 mM, and quercetin: water solubility 60.0 mg/dm$^3$, 0.2 mM at 16 °C), the production of carrier systems can help to overcome this drawback by encapsulation of these compounds in lipid-based systems (emulsions, nanoparticles, micelles or liposomes) [16,17,18]. Liposomes are suitable for use as delivery systems in biological systems and to ensure a good cellular uptake [19,20,21,22].

The phospholipid bilayers of liposomes surround an aqueous environment suitable for drugs loading in order to have a targeted release. Liposomes composition, charge and size are important factors for both efficacy and adverse effects [23,24,25,26,27]. The study of liposomes with different superficial charge allows to evaluating chemical and physical properties that can affect both encapsulation and release processes of the two antioxidants. In vitro study, the liposome surface charge influence many activities such as the binding and endocytosis of interactions [28]. Cationic liposomes induced apoptosis in mouse splenic macrophages and macrophage-like cell [29,30], whereas the use of anionic liposomes has been fairly restricted to the delivery of specific therapeutic macromolecules [31] influencing many aggregation or absorption phenomena [32].

On the basis of these reasoning a series of liposomes encapsulating quercetin and rutin, have been synthesized, chemically characterized and evaluated as regards the correlations between chemical properties and biological responses.

Two important points were considered in this paper: (1) the choice of two natural active compounds having a similar chemical structure (quercetin and rutin) whose physic-chemical characteristics may affect the biological response; (2) the synthesis and therefore the use, of different surface charged liposomes (anionic, cationic and zwitterionic or non-ionic) as preliminar step for setting down the best drug delivery system for natural products. The different surface charge may be strongly correlated to toxicity, cell growth, etc.

Three different lipid compositions, varying the surface charge (zwitterionic, cationic and anionic) have been used to synthesize liposomes, that are then evaluated for in vitro cytotoxicity towards fibroblasts NIH3T3. Moreover, the liposomes, loaded with both quercetin and rutin, have been tested for the ability to protects cells against hydrogen peroxide-induces oxidative, in comparison with free rutin and quercetin solution.

The main purpose was to evaluate the protective effects on oxidative stress phenomena that different natural products loaded into liposomes could have on stabilized cell lines.

## 2. Material and methods

### 2.1. Materials

Quercetin (Q) and rutin (R) were purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland) and used without further purification. Stock solutions in ethanol were stored at − 20 °C. DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine), DOPE (1,2-di-(9Z-octadecenoyl)-*sn*-glycero-3-phosphoethanolamine), DOPA (1,2-dioleoyl-*sn*-glycero-3-phosphate) and DOTAP (1,2-dioleoyl-3-trimethylammonium-propane) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Medium 131 was obtained from Invitrogen (USA). Dulbecco's Modified Eagle's Medium (DMEM), trypsin solution, and all the solvents used for cell culture were purchased from Lonza (Belgium). Mouse immortalized fibroblasts NIH3T3 were from American Type Culture Collection.

### 2.2. Quercetin and rutin loaded liposomes preparation

DOPC/DOPE, DOPE/DOPA, and DOTAP/DOPE were prepared at 1:1 M ratio with a final total lipid concentration of $1.0 \times 10^{-2}$ M. Quercetin and rutin loaded liposomes were prepared to obtain a final 1:1 M ratio between total lipids and antioxidants.

Liposomes were prepared in a round bottom vial by mixing appropriate amounts of stock solutions, which were $4.0 \times 10^{-2}$ M in chloroform for lipids, and $2.0 \times 10^{-2}$ M in ethanol for Q and R. A dry lipid film (with and without antioxidants) was obtained by evaporating the solvent under vacuum overnight. Rehydrating with Milli-Q grade $H_2O$ yielded multilamellar dispersion.

Upon vortexing, multilamellar vesicles were obtained, which were then submitted to eight freeze/thaw cycles. This method improved the homogeneity of the size distribution in the final suspension. Liposomes were subsequently reduced in size and converted to unilamellar vesicles by extrusion through 100 nm polycarbonate membranes. Twenty-seven extrusions were performed with the LiposoFast apparatus (Avestin, Ottawa, Canada). All liposomes were stored at 4 °C.

### 2.3. Size and surface charge of liposomes

Dynamic Light Scattering (DLS) and ζ–potential measurements were performed on a Zeta-sizer Instrument Nano ZS90 (Malvern Instrument Ltd., UK).

ζ-potential values were measured at 25 °C. As the radii of liposomes were always large enough compared with the Debye–Huckel parameters, the ζ-potentials were calculated directly from the Helmoholtz–Smolowkovski equation (by the zetasizer) [33]. Sizes were