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Olive Oil-Based Delivery of Photosensitizers for Bacterial Eradication

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1. Introduction

Olive oil is a natural product of *Olea europaea*. It contains triacylglycerols of unsaturated and saturated fatty acids as well as free acids and numerous other biologically active components. Modern pharmaceutical industries are turning to natural herbal sources in order to find effective, low allergenic and non-irritating components that can be used in drug delivery systems or as recipients for both hydrophobic and hydrophilic active agents. Combining hydrophobic compounds with olive oil components is not problematic at all. However, this is quite different for hydrophilic compounds. One possible way for overcoming this problem is by mechanochemical treatment. This method has become widespread for preparing powdered solid materials in a large variety of compositions and involves the use of a conventional high-energy ball mill to initiate chemical reactions and structural changes of materials in solid-phase processes. Mechanochemical activation appears to be an environmentally friendly method, since it does not require organic solvents (Grigorieva et al., 2004; Margetić, 2005; Lugovskoy et al., 2008; Lugovskoy et al., 2009). It was shown that the mechanochemical method enabled some olive oil components to covalently attach to talc or to titanium dioxide - the solid ingredients of creams, ointments and powders (Nisnevitch et al., 2011). The remaining components were deeply absorbed by solid phases. New solid-phase composite materials which combined useful properties of various components with a different nature were thus created. Talc combined with olive oil exhibited good antioxidant properties scavenging ca. 40% of free radicals. Olive oil phenols with one or two hydroxyl groups, such as hydroxytyrosol, caffeic acid, photocatechuic acid, syringic acid, derivatives of elenolic acid, derivatives of oleuropein, tyrosol and some others are among the olive oil components responsible for its *in vitro* antioxidative activity (Papadopoulos & Boskou 1991; Briante et al., 2001; Lesage-Meessen et al., 2001; Tovar et al., 2001; Vissers et al., 2004). These compounds retain their antioxidant properties when combined with talc by a mechanochemical method. Furthermore, the possibility of combining water-soluble ascorbic acid (vitamin C) with olive oil on a talc or titanium dioxide support using mechanochemical activation has been reported (Nisnevitch et al., 2011). These triple mixtures (support-olive oil-ascorbic acid) scavenged free radicals instantly and totally due to the presence of ascorbic acid, which is a well-known effective

antioxidant (Cathcart, 1985). The scavenging ability in the triple mixtures after mechanochemical treatment was as good as that of the double mixtures of ascorbic acid with the supports. Mechanochemical inclusion of ascorbic acid into composites of olive oil with talc or olive oil with titanium dioxide successfully combined hydrophobic and hydrophilic components and provided high antioxidant properties to the entire system despite the covalent bonding between the components (Nisnevitch et al, 2011).

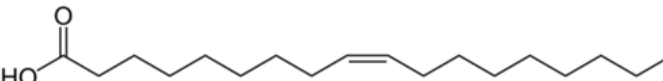
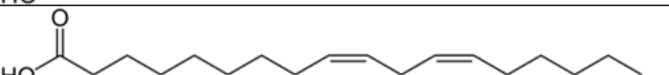


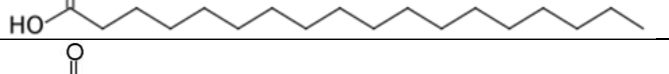
New olive oil-based composite materials exhibit pronounced bactericidal properties. The antimicrobial activity of the mechanochemically treated triple mixtures which were pressed into pellets was examined against the Gram-positive *S. aureus* and the Gram-negative *E. coli* bacteria. Samples containing ascorbic acid on a titanium dioxide support were more effective against both bacteria than a talc support, probably because of weaker bonding of ascorbic acid to titanium dioxide than to talc, which contributed to better diffusion of the ascorbic acid out of the pellets. Gram-positive *S. aureus* was more sensitive to all the ascorbic acid-containing samples than the Gram-negative *E. coli*, but *E. coli* responded to addition of olive oil into both talc-ascorbic acid and titanium dioxide-olive oil mixtures. In the latter case, the inhibitory activity of the triple composites was higher than that of double ascorbic acid-support composites. The antimicrobial activity of all the ascorbic acid-containing samples depended on the ascorbic acid content in the pellets. Olive oil, olive fruit and olive leaf extracts are known to exhibit a broad antimicrobial, antimycoplasmal and antifungal spectrum due to the presence of long chain α,β -unsaturated aldehydes, phenolic glycoside oleuropein and several other phenol compounds (Fleming et al. ,1973; Kubo et al., 1995; Bisignano et al., 2001; Furneri et al., 2002; Medina et al., 2007; Covas et al., 2009; Kampa et al., 2009). Mechanochemical combination of natural antimicrobial agents from olive oil with ascorbic acid, which is a strong bacterial suppressor, enabled the production of highly active solid-phase antibacterial composites.

Hydrophilic and hydrophobic components can also be combined by encapsulating hydrophilic constituents in lipid vesicles called liposomes. Such lipid-based formulations are actually possible carriers for both hydrophobic and hydrophilic active components and can be applied as drug delivery systems. Liposome formulations possess enhanced abilities to penetrate the skin, thus improving the delivery process. Lipid-based drug administration can increase treatment efficiency in cases of skin infections and inflammations caused by bacterial invasion.

2. Olive oil-containing liposomes

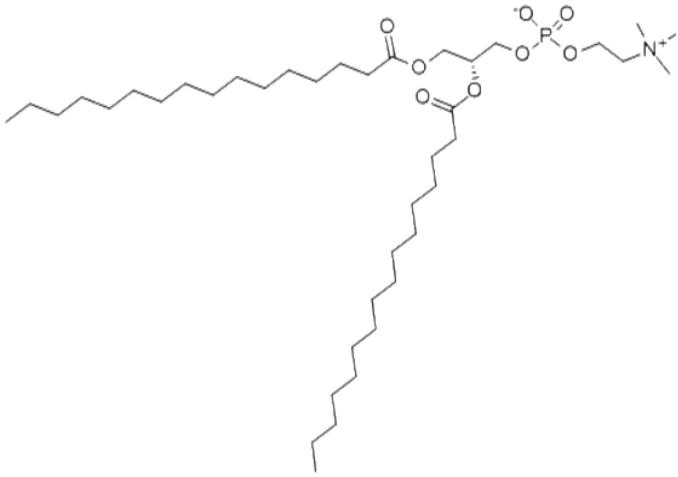
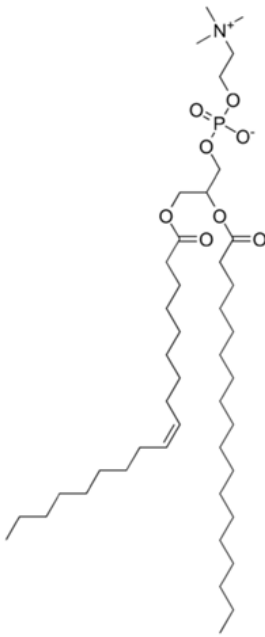
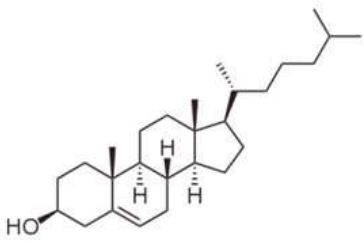
Liposomes (nano or micro-scale vesicles) can be obtained using phospholipids' property of self-assembly in the presence of an aqueous phase. Phospholipids spontaneously form a closed spherical phospholipid bilayer such that phosphate groups are in contact with the aqueous phase on the internal and external surfaces, and lipid chains are hidden within the membrane. Such a phospholipid assembly results in large multilamellar liposomes, which are constructed from alternating concentric lipid and aqueous layers. Treatment of multilamellar liposomes by ultrasound, membrane extrusion or other methods leads to the formation of unilamellar liposomes which consist of a single lipid bilayer (Chrai et al., 2001). Liposomes are convenient carriers of both hydrophilic and hydrophobic molecules, where the former can be incorporated into aqueous layers of multilamellar liposomes or

$$\begin{array}{c} \text{H} & & \text{O} \\ | & & || \\ \text{H} - \text{C} - \text{O} - \text{C} - \text{R} \\ | & & \text{O} \\ & & || \\ \text{H} - \text{C} - \text{O} - \text{C} - \text{R} \\ | & & \text{O} \\ & & || \\ \text{H} - \text{C} - \text{O} - \text{C} - \text{R} \\ | \\ \text{H} \end{array}$$

Fatty acid	Structure	% in Virgin Olive Oil (Hatzakis et al., 2008)		% in EPC (Ternes, 2002; Sigma aldrich.com)	% in DPPC (northern lipids.com)
		Triacylglycerols	Phospho-lipids		
Oleic acid		72.0-81.6	72.5-82.9	26-31	-
Linoleic acid		4.6-11.0	2.7-12.0	13-19	-
Palmitic acid		12.3-19.7	11.2-19.4	27-33	100
Stearic acid				13-15	-
α -Linolenic acid		0.08-0.53	0.11-0.47	0-0.2	-

Liposomes can be exploited as carriers for controlled drug delivery and targeting to cells. Liposome formulations of drugs have several advantages over the use of drugs in their free form: liposomes guarantee delivery of a highly concentrated drug, liposomes protect the drugs from degradation during the delivery process, liposomes are applicable for polar as well as for nonpolar drugs, and ingredients of the liposomes themselves are nontoxic and biodegradable (Chrai et al., 2002). Liposome components participate in drug delivery, but not in drug function, such that liposomes actually play the role of excipients (Chen, 2008). Additional ingredients can be incorporated into the phospholipid bilayer in order to impart needed properties to liposomes, as indicated by the following examples: negatively charged phosphatidylinositol or positively charged stearylamine can be incorporated into the phospholipid bilayer in order to obtain charged liposomes (Robinson et al., 2001); addition of cholesterol provides rigidity to the liposome structure (New, 1994). The latter example is

explained by an increase in the gel-to-liquid crystalline phase transition temperature (T_c) of the lipid liposome layer upon the addition of cholesterol (Beaulac et al., 1998).

Component	Structure
Dipalmitoyl phosphatidylcholine (DPPC)	
Egg phosphatidylcholine (EPC)*	
Cholesterol	

*Alternative fatty acids residues are listed in the Table 1.

Table 2. Compounds used as a basis for liposome preparations.

The major ingredients of olive oil are triacylglycerols (Fig. 1) of unsaturated and saturated fatty acids (Table 1), mainly of oleic acid, but it also contains mixed triacylglycerols of palmitic-oleic-oleic, linoleic-oleic-oleic, palmitic-oleic-linoleic, stearic-oleic-oleic, linolenic-oleic-oleic and other acids (Nichols & Sanderson, 2002; oliveoilsource.com).

Olive oil also contains a small amount of free fatty acids and several minor constituents necessary for health – tyrosol, hydroxytyrosol and their derivatives such as oleuropein, oleuropein aglycone, dialdehydic form of oleuropein aglycone, decarboxymethyl form of oleuropein aglycone and ligstroside aglycone; phenolic acids, for example, 4-hydroxybenzoic acid, protocatechuic acid, syringic acid and 4-hydroxy-phenylacetic acid; flavonoids and lignads, for instance, apigenin, luteolin, pinoresinol and acetopinoresinol; squalene, α -tocopherol, vitamins E and K, pigments chlorophyll, pheophytin, carotenoids and other compounds (Boskou et al., 2006a,b; Boskou 2009a,b). In addition, olive oil includes phospholipids at a concentration range of 11-157 mg/kg in virgin olive oil (Hatzakis et al., 2008) and 21-124 mg/kg in cloudy (veiled) virgin olive oil (Koidis & Boscou, 2006).

Component	DPPC liposomes, % (w/w)	EPC liposomes % (w/w)
Dipalmitoyl phosphatidylcholine	62	-
Egg phosphatidylcholine	-	64
Olive oil	30	28
Cholesterol	8	8

Table 3. Weight compositions of olive oil based liposomes.

The liposomes used in this work were composed of dipalmitoyl phosphatidylcholine (DPPC, Northern Lipids Inc., Canada) or egg yolk phosphatidylcholine (EPC, Sigma, USA) also named L- α -lecithin. These phospholipids are constructed based on the phosphatidylcholine. However, DPPC has a homogeneous composition and contains only saturated palmitic acid residues, contrary to the heterogeneous composition of EPC, which includes several saturated and unsaturated fatty acid residues (Table 1). EPC liposomes are composed of two different fatty acid residues, where one residue is usually saturated and the other is unsaturated, as demonstrated in Table 2 (Kent & Carman, 1999). The most common fatty acids incorporated into the EPC structure are presented in Table 1. Phosphatidylcholine, the major membrane phospholipid in eukaryotic cells, is the source of the bioactive lipids lysophosphatidylcholine, phosphatidic acid, diacylglycerol, lysophosphatidylcholine, platelet activating factor and arachidonic acid. It also plays a role as a reservoir for several lipid messengers (Kent & Carman, 1999).

We incorporated virgin olive oil (Yad Mordechai, Israel) into the lipid bilayer in order to enhance the biocompatibility of liposomes and enrich them with natural salubrious components. For this purpose, organic solutions of DPPC or EPC together with olive oil were prepared, and the organic solvent was evaporated in a round-bottom flask to dryness in a vacuum rotary evaporator to obtain a thin lipid film which was vigorously agitated with buffer solutions with or without water-soluble active agents.

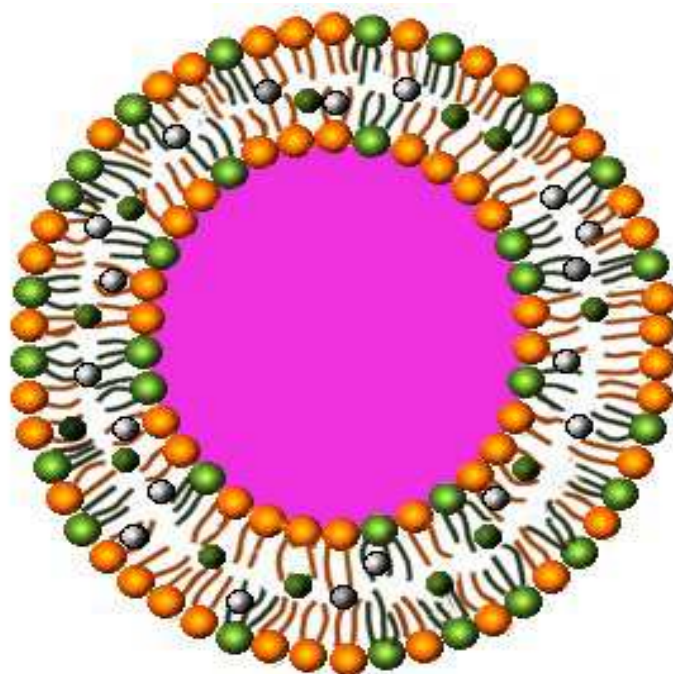


Fig. 2. A schematic representation of unilamellar olive oil including liposome. Phospholipid molecules are orange coloured, olive oil components are green, cholesterol molecules are grey coloured. Internal liposome volume containing water-soluble active components is pink coloured.

As can be seen from Table 1, both used by us phospholipids are built from the same fatty acids as olive oil triacylglycerols and olive oil phospholipids, although in different proportions. This fact points to high compatibility between the used phospholipids and olive oil. Various combinations of phospholipids and olive oil were attempted, and it was found that a homogeneous lipid film could not be obtained with any combination of olive oil and EPC and at a high olive oil content added to DPPC. A small amount of cholesterol (Table 2) was added to the lipid mixture solution in order to increase the lipid film's rigidity. Even and homogeneous films were attained after this addition, which resulted in stable liposomes. The multilamellar liposomes were transformed into unilamellar liposomes by sonication, as described previously (Nisnevitch et al., 2010; Nakonechny et al., 2010). Final compositions found to be appropriate for liposome preparation are presented in Table 3.

A schematic representation of the olive oil-based unilamellar liposomes is presented in Fig. 2. Triacylglycerol olive oil components are organically incorporated into the phospholipid-based structure, hydrophobic olive oil constituents such as polyphenols or vitamins are incorporated into the liposome bilayer and the aqueous solution is located in the inner liposome space. The prepared liposomes were used for encapsulation of active bactericidal factors as described in part 4.

The prepared olive oil-based liposomes were characterized by average size, evaluated by measuring the turbidity spectra. This method is based on the determination of an equation of the turbidity spectra curves, estimation of the power " n " in the equation (1):

$$\log \frac{I_o}{I} = K \lambda^{-n}$$

(1)

where $\log \frac{I_o}{I}$ - a measured turbidity value, I_o - initial light intensity, I - light intensity and λ - wavelength, and the liposome average size evaluation with a calibration curve representing "n"-values' dependence on vesicle sizes (Trofimov & Nisnevich, 1990; Nisnevitch et al, 2010). Higher "n"-values correspond to smaller vesicle sizes. Turbidity spectra of DPPC and EPC liposomes with and without addition of cholesterol and olive oil were measured (Fig. 3), and corresponding type (1) equations were found in each case. As can be seen from Table 4, "n" values in these equations and correspondingly, vesicle sizes, are different for DPPC and EPC liposomes, and vary when cholesterol and olive oil are incorporated into the phospholipid layers.

As can be seen from Table 4, the DPPC-based liposomes are smaller than the EPC ones obtained using the same treatment conditions. This phenomenon can be explained by two factors - by the lipid structure and by the lipid phase state of the liposomes. The homogeneous composition of DPPC, which contains only saturated palmitic acid residues, enables dense lipid packing in the liposome bilayers, in contradistinction to the heterogeneous composition of EPC, which includes several saturated and unsaturated fatty acid residues (Table 1). Such a denser package leads to the formation of unilamellar DPPC liposomes with a smaller diameter. At the temperatures of our experiments (from room temperature to 37°C), DPPC exists in a gel phase state (T_c of DPPC is 41°C (avantilipids.com)), whereas EPC is found in a liquid crystal state (T_c of EPC is -10°C (Kahl et al., 1989)). Acyl chains of phospholipids are more disordered and bulky in a fluid state, thus causing an increase in surface area per phospholipid molecule which results in bigger liposomes in the case of EPC liposomes (New, 1994).

Liposome composition	DPPC-based liposomes		EPC-based liposomes	
	"n"-value in equation (1)	Vesicle size, nm	"n"-value in equation (1)	Vesicle size, nm
Phospholipid alone	2.44	200	1.57	> 400
Phospholipid and cholesterol	2.50	190	2.00	> 400
Phospholipid, cholesterol and olive oil	2.03	> 400	1.36	> 400

Table 4. Turbidity spectra parameters and vesicle size for liposomes of various compositions.

Addition of cholesterol to phospholipids resulted in an increase in the "n"-value, which means that the vesicle size decreased upon the addition of cholesterol. The liposome size increased again after olive oil was incorporated into the membrane structure (Table 4). These facts can be explained by taking the correlation between liposome rigidity and size into account. Addition of cholesterol caused the liposome vesicles to become more rigid and respectively smaller, and further addition of olive oil led to disturbance of the lipid layer and to an increase in size (Table 4).

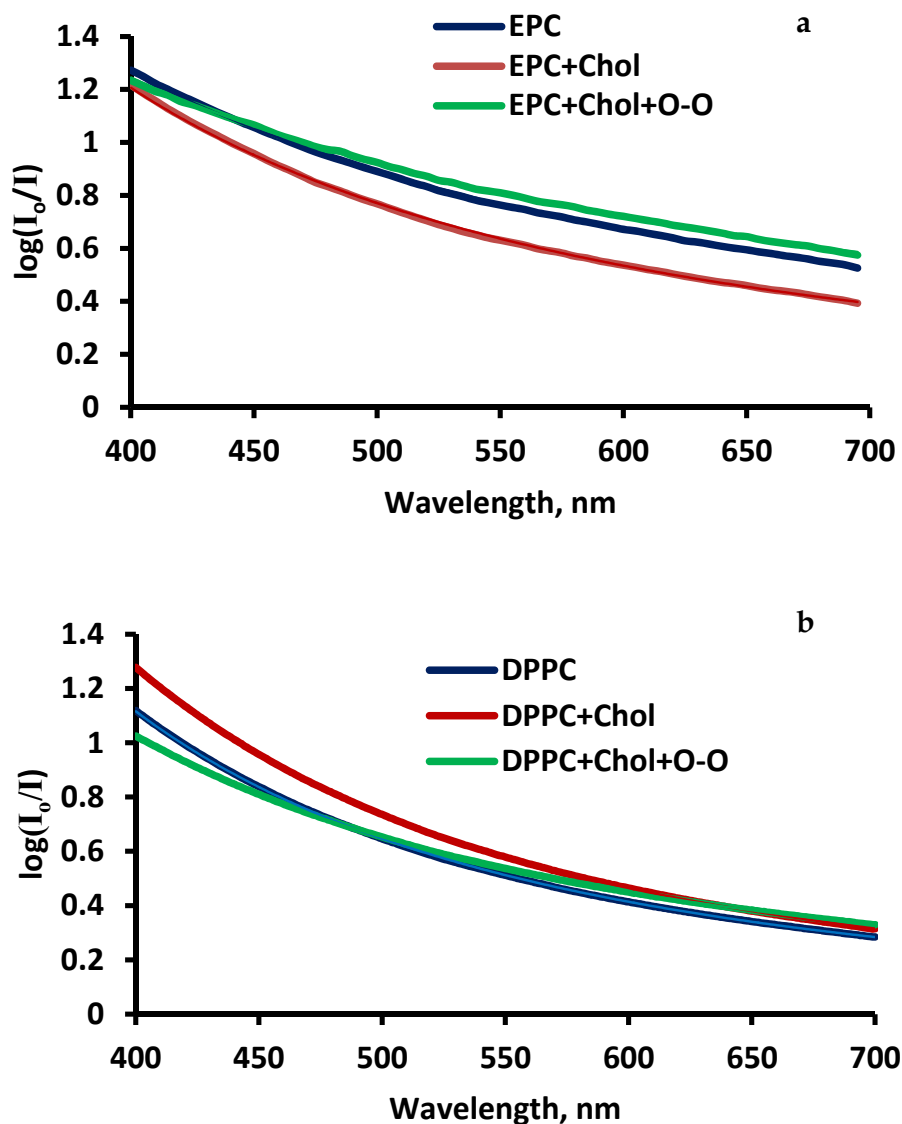


Fig. 3. Turbidity spectra of liposomes with and without additions of cholesterol (Chol) and olive oil (O-O). a – EPC-based liposomes, b – DPPC- based liposomes.

3. Photosensitizers encapsulated in olive oil-containing liposomes

Bacterial resistance to antibiotics has become a serious problem worldwide, causing an urgent need to develop new approaches and ways to overcome the evolution and spread of drug-resistant strains (Patterson, 2006; Maragakis et al., 2008; Moellering et al., 2007). One alternative to treatment of infections by antibiotics is photodynamic antimicrobial chemotherapy (PACT), which is based on the use of non-toxic compounds – photosensitizers, which can be activated by visible light. Excited photosensitizer molecules return to a ground level by transferring their energy to dissolved molecular oxygen with production of reactive oxygen species, which leads to direct damage of cellular components (Macdonald & Dougherty, 2001; Wainwright, 1998). This process is explained in Fig. 4.

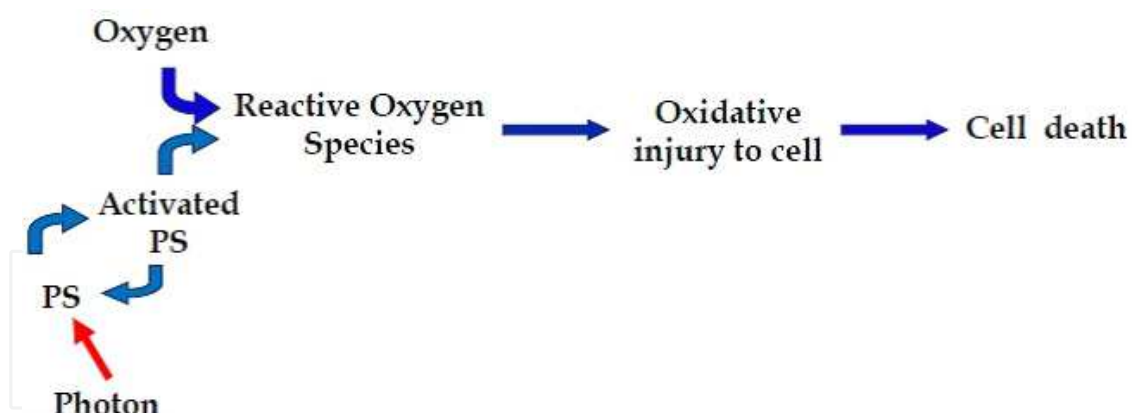


Fig. 4. A scheme of photosensitizer (PS) activation upon illumination which visible light and its cytotoxic action.

Photosensitizers refer to several chemical groups - porphyrins, phenothiazinium, phthalocyanines, xanthenes, chlorin derivatives and others. However, a feature common to all of these groups is the presence of conjugated double bonds, which allow effective absorbance of light energy. The history, mechanism of action and biomedical applications of PACT have been reviewed extensively (Nitzan & Pechatnikov, 2011; Malik et al., 2010; Reddy et al., 2009; Randie et al., 2011; Daia et al., 2009). Two photosensitizers, Rose Bengal and Methylene Blue, were used in this work. Rose Bengal relates to a xanthene (halogenated xanthenes) group of photosensitizers, and is negatively charged under physiological conditions. Methylene Blue represents a phenothiaziniums group and exists in cationic form. The structures of these compounds are shown in Fig.5.

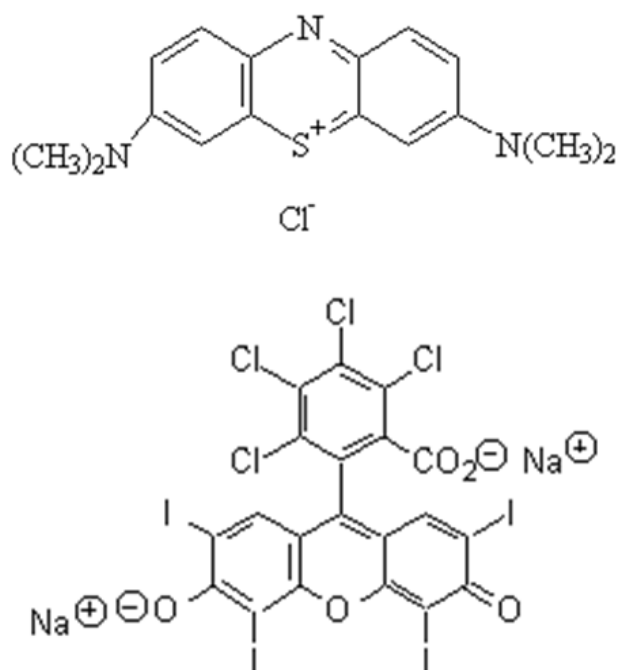


Fig. 5. Structures of photosensitizers Methylene Blue (upper) and Bengal Rose (lower).

Both photosensitizers absorb visible light, and their absorption spectra are presented in Fig. 6.

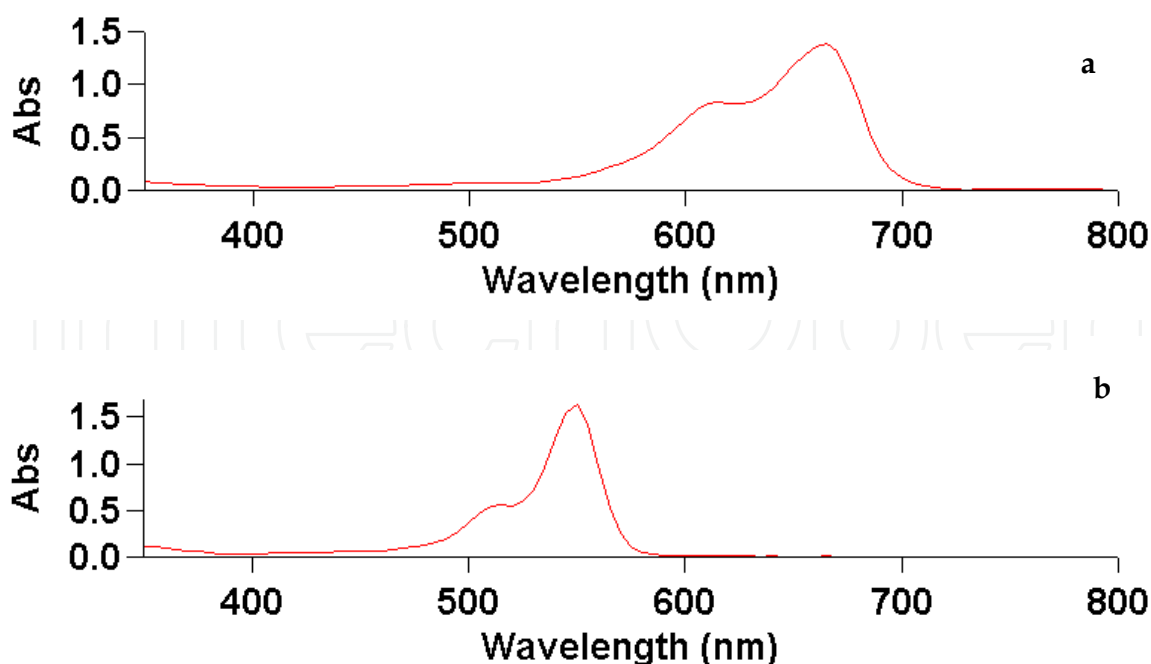


Fig. 6. Absorption spectra of (a) Methylene Blue and (b) Bengal Rose.

The described photosensitizers were encapsulated into DPPC and EPC liposomes with and without addition of olive oil as previously described by us (Nisnevitch et al., 2010). Liposomes with encapsulated photosensitizers were separated from free photosensitizers by centrifugation, and absorption of free photosensitizers was measured at the appropriate wavelengths (665 nm for Methylene Blue and 550 nm for Rose Bengal, Fig. 6).

$$\frac{A_0 \cdot V_0 - A \cdot V}{A_0 \cdot V_0} \cdot 100\% \quad (2)$$

where - A_0 - absorbance of the initial photosensitizer in the volume V_0 and A - absorbance of the free photosensitizer in the volume V . The encapsulation rate reached $50 \pm 5\%$ in all cases.

The extent of the photosensitizers encapsulation in liposomes was estimated by formula (2) as the ratio of the encapsulated photosensitizer amount, taken as the difference between initial and free photosensitizer amount, and the initial amount.

4. Bactericidal properties of photosensitizers encapsulated in olive oil-based liposomes

Application of liposomal forms of various drugs is widely used in cases of cancer and bacterial infection treatment. Treatment of tumours by liposomal forms of doxorubicin led to a manifold accumulation of the drug in the malignant cells (Drummond et al., 1999). Entrapment of photosensitizers into liposomes was also successfully applied for eradication of cancer cells (Derycke & de Witte, 2004). Liposome-encapsulated tobramycin, unlike its free form, was demonstrated to be highly effective against chronic pulmonary *P. aeruginosa* infection in rats (Beaulac et al., 1996). Drug administration using liposomes provided a delivery of active components in a more concentrated form and contributed to their

enhanced cytotoxicity. A mechanism of drug delivery by liposomes was examined for Gram-negative and Gram-positive bacteria. Gram-negative and Gram-positive bacteria differ in their cell wall structure. Gram-negative cells possess an outer membrane which contains phospholipids, lipoproteins, lipopolysaccharides and proteins, peptidoglycan and cytoplasmic membrane. Gram-positive bacteria do not have an outer membrane, and their cell wall consists of peptidoglycan and an inner cytoplasmic membrane (Baron, 1996).

In Gram-negative bacteria, fusion between drug-containing liposomes and the bacterial outer membranes occurs, which results in the delivery of the liposomal contents into the cytoplasm. This mechanism was verified by scanning electron microscopy (Mugabe et al., 2006; Sachetelli et al., 2000), and it is schematically shown on the Fig. 7a.

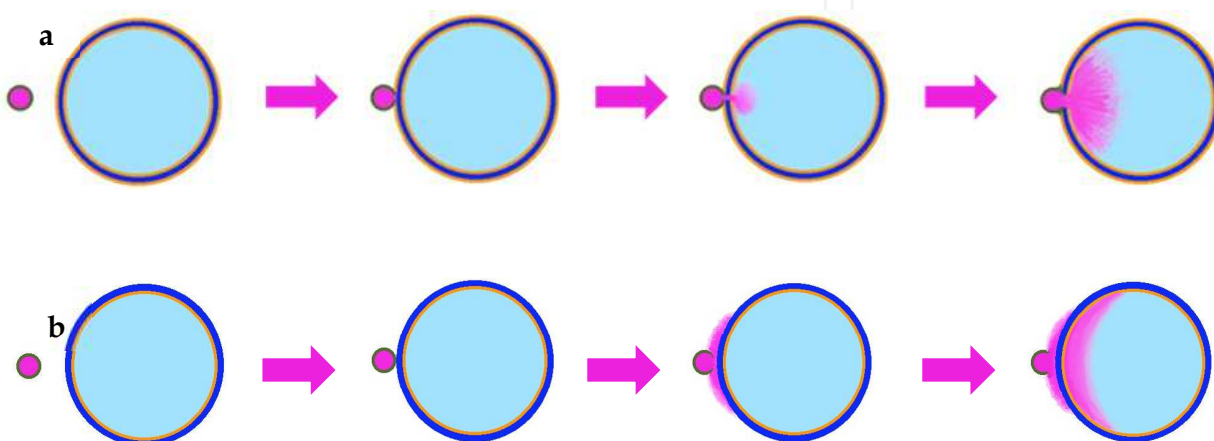


Fig. 7. A schematic representation of liposome-encapsulated drug delivery to (a) Gram-negative and (b) Gram-positive bacteria cells.

In Gram-positive bacteria, liposomes are assumed to release their content after interaction with the external peptidoglycan barrier, enabling passive diffusion through the cell wall (Furneri et al., 2000). This drug delivery mechanism is demonstrated in Fig. 7b. Application of liposomal forms of drugs leads to prolongation of their action in infected tissues and provides sustained release of active components (Storm & Crommelin, 1998).

Gram-positive and Gram-negative bacteria respond differently to PACT, with the former being more susceptible to the treatment. Gram-negative bacteria do not bind anionic photosensitizers (Minnock et al., 2000), unless additional manipulations facilitating membrane transport are used (Nitzan et al., 1992), due to the more complex molecular and physico-chemical structure of their cell wall. PACT is considered to have good perspectives in the control of oral and otherwise localized infections (Meisel & Kocher, 2005; O'Riordan et al., 2005). Local application of liposome-entrapped drugs can prolong their action in infected tissues and provide sustained release of active components (Storm & Crommelin, 1998). It should be mentioned that bacterial resistance to photosensitizers has not been reported to date.

Liposome formulations of photosensitizers showed high efficiency in eradication of both Gram-negative and Gram-positive bacteria. Liposome or micelle-entrapped hematoporphyrin and chlorin *e6* were found to be effective against several Gram-positive bacteria, including methicillin-resistant *S. aureus* (Tsai et al., 2009).

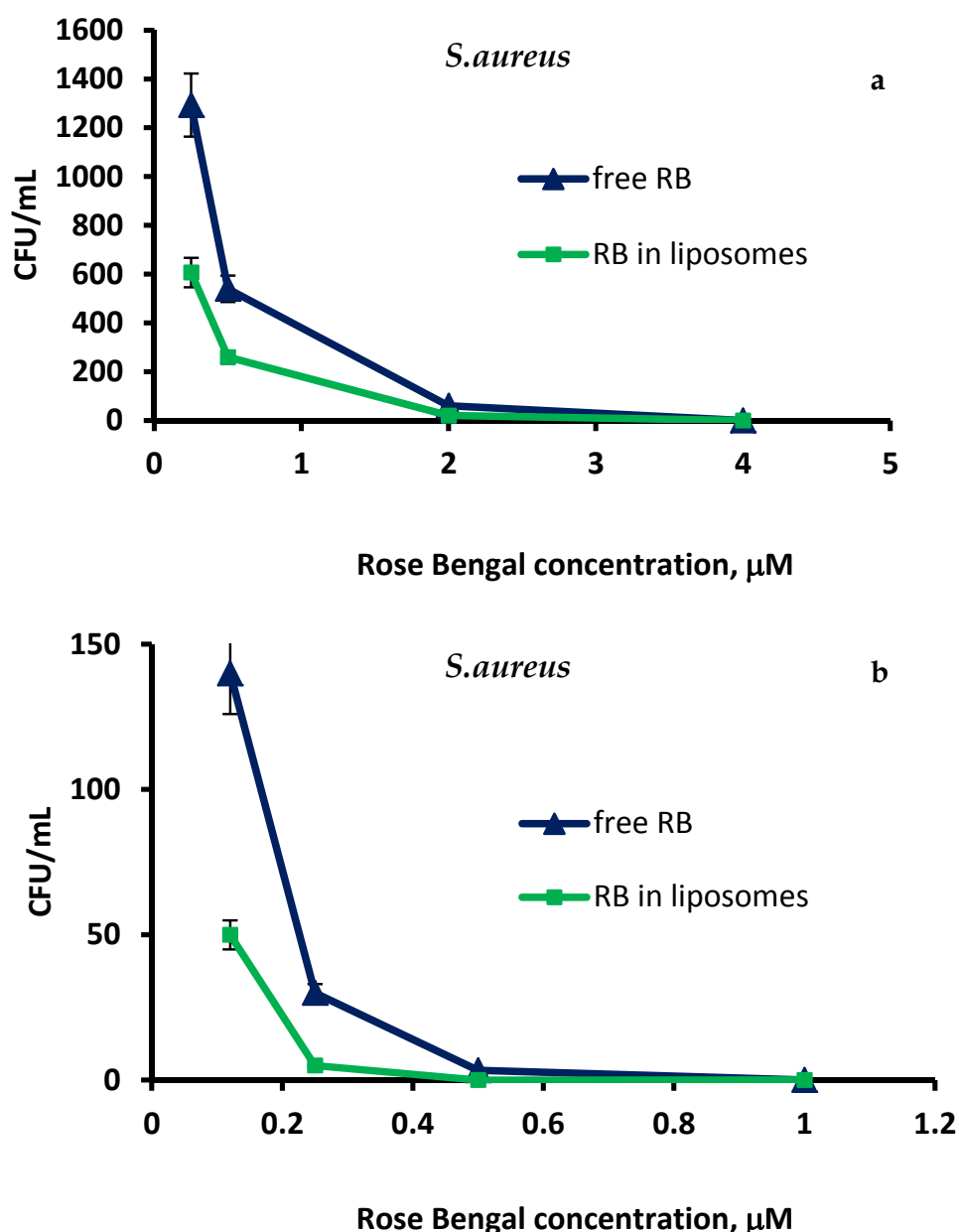


Fig. 8. Eradication of *S. aureus* by various concentrations of Rose Bengal (RB) in a free form and encapsulated into EPC-olive oil liposomes under white light illumination at initial bacteria concentration of (a) $3 \cdot 10^9$ cells/mL and (b) $3 \cdot 10^7$ cells/mL.

Encapsulation of photosensitizers into liposomes does not always result in enhancement compared to the free-form cytotoxic activity. The activity of m-tetrahydroxyphenylchlorin in liposomal form was comparable to the free form activity of PACT inactivation of a methicillin-resistant *S. aureus* strain (Bombelli et al., 2008). When tested against methicillin-resistant *S. aureus*, chlorophyll *a* was reported to be more efficient in free form than in a liposomal formulation, whereas hematoporphyrin as well as a positively charged PS 5-[4-(1-dodecanoylpyridinium)]-10,15,20-triphenyl-porphyrin were less effective in free form than upon encapsulation in liposomes. These results were explained by differences in photosensitizer chemistry which may influence their association with liposomal components, lipid fluidity and localization in liposome vesicles (Ferro et al., 2006; 2007).

We have previously shown that Methylene Blue encapsulated in liposomes composed of DPPC or EPC effectively deactivated several Gram-positive and Gram-negative bacteria, including *S. lutea*, *E. coli*, *S. flexneri*, *S. aureus* and MRSA, and that liposomal Rose Bengal also eradicated *P. aeruginosa* (Nisnevitch et al., 2010; Nakonechny et al., 2010; 2011).

Olive oil-containing liposomes loaded with photosensitizers were tested for their antimicrobial activity under white light illumination against two Gram-positive bacteria of the genus *Staphylococcus* – *S. aureus* and *S. epidermidis*. Although *S. epidermidis* is part of the normal skin flora, it can provoke skin diseases such as folliculitis, and may cause infections of wounded skin, in particular around surgical implants. *S. aureus* is defined as a human opportunistic pathogen and is a causative agent in up to 75% of primary pyodermas, including carbuncle, ecthyma, folliculitis, furunculosis, impetigo and others (Maisch et al., 2004).

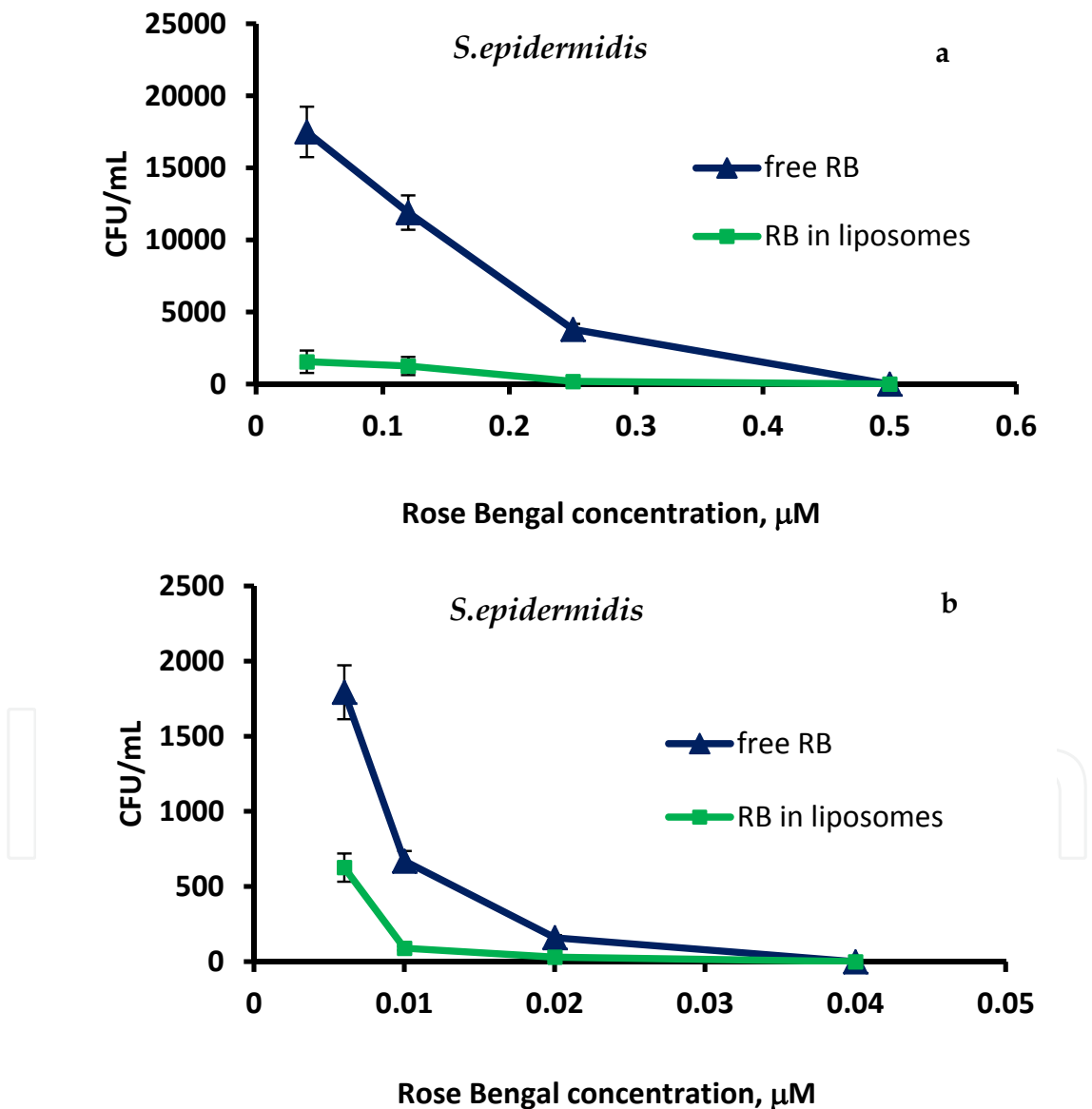


Fig. 9. Eradication of *S. epidermidis* by various concentrations of Rose Bengal (RB) in a free form and encapsulated into EPC-olive oil liposomes under white light illumination at initial bacteria concentration of (a) 3·10⁸ cells/mL and (b) 3·10⁶ cells/mL.

The water-soluble photosensitizers Rose Bengal and Methylene Blue were encapsulated in the above-described unilamellar liposomes at various concentrations and were examined under white light illumination against various cell concentrations by a viable count method as described previously (Nakonechny et al., 2010) and the number of bacterial colony forming units (CFU) was determined. This number characterized the concentration of bacterial cells which survived after a treatment.

The antimicrobial effect of liposomes incorporated with olive oil and loaded with Rose Bengal was strongly dependent on its concentration (Fig. 8 and 9). As can be seen from Fig. 8a, treatment of *S. aureus* with EPC-based liposomes caused a million-fold suppression of the bacterial cells at 0.25 μM of Rose Bengal and total eradication at a concentration of 2 μM when tested at an initial cell concentration of $3 \cdot 10^9$ cells/mL. Total eradication of *S. aureus* at an initial concentration of $3 \cdot 10^7$ cells/mL occurred already at a liposome-encapsulated Rose Bengal concentration of 0.5 μM (Fig 8b).

A principal similar trend was observed for *S. epidermidis*. It was necessary to apply liposome-encapsulated Rose Bengal at a concentration of 0.25 μM for total eradication of bacteria at an initial concentration of $3 \cdot 10^8$ cells/mL (Fig. 9a), and it was enough to apply 0.02 μM encapsulated photosensitizer for killing bacteria at $3 \cdot 10^6$ cells/mL (Fig. 9b). *S. epidermidis* exhibited a higher sensitivity than *S. aureus* for the liposome formulation of Rose Bengal compared with its free form. For *S. aureus*, liposomal Rose Bengal was only twice as effective as its free form – at each Rose Bengal concentration its liposomal form caused two-fold higher suppression of the bacteria. In contradistinction, *S. epidermidis* was suppressed three to twelve times more effectively by Rose Bengal encapsulated in liposomes than by the free photosensitizer.

Bacterial eradicating ability of the encapsulated as well as of the free Rose Bengal was demonstrated to depend on the initial concentration of the bacteria. When tested at the same Rose Bengal concentration, a suppression of both bacteria varied from partial to total. As can be seen from Fig. 10a, a 0.25 μM concentration of Rose Bengal encapsulated in EPC-olive oil liposomes caused a decrease of up to $6 \cdot 10^2$ cells/mL in the *S. aureus* concentration when taken at an initial concentration of $3 \cdot 10^9$ cells/mL (corresponding to 6.7 \log_{10} CFU/mL) and up to zero cell concentration when taken at $3 \cdot 10^7$ or $3 \cdot 10^6$ cells/mL. In the case of *S. epidermidis*, 0.01 μM encapsulated Rose Bengal induced bacterial reduction of up to $1.5 \cdot 10^4$ cells/mL from the initial concentration of 10^8 cells/mL, and to the zero concentration at an initial concentration of $3 \cdot 10^6$ cells/mL (Fig. 10b).

DPPC-based liposomes were also examined, in addition to EPC-based olive oil-containing liposomes. The results showed high antimicrobial efficiency of the olive oil-containing liposomes in both bases, which was not less than that of the liposomes without olive oil supplements. Fig. 11 relates to the antimicrobial activity of Rose Bengal, applied against *S. epidermidis*, in free form or encapsulated in olive oil-containing ECP- and DPPC-liposomes, as well as to EPC-liposomes without olive oil. The data presented in Fig. 11 indicate that at each initial concentration, all liposomal forms of Rose Bengal eradicated bacteria more effectively than its free form (P-value 0.015), but there was no statistically significant difference in the photosensitizer activity when encapsulated in various types of liposomes (P-value 0.86).

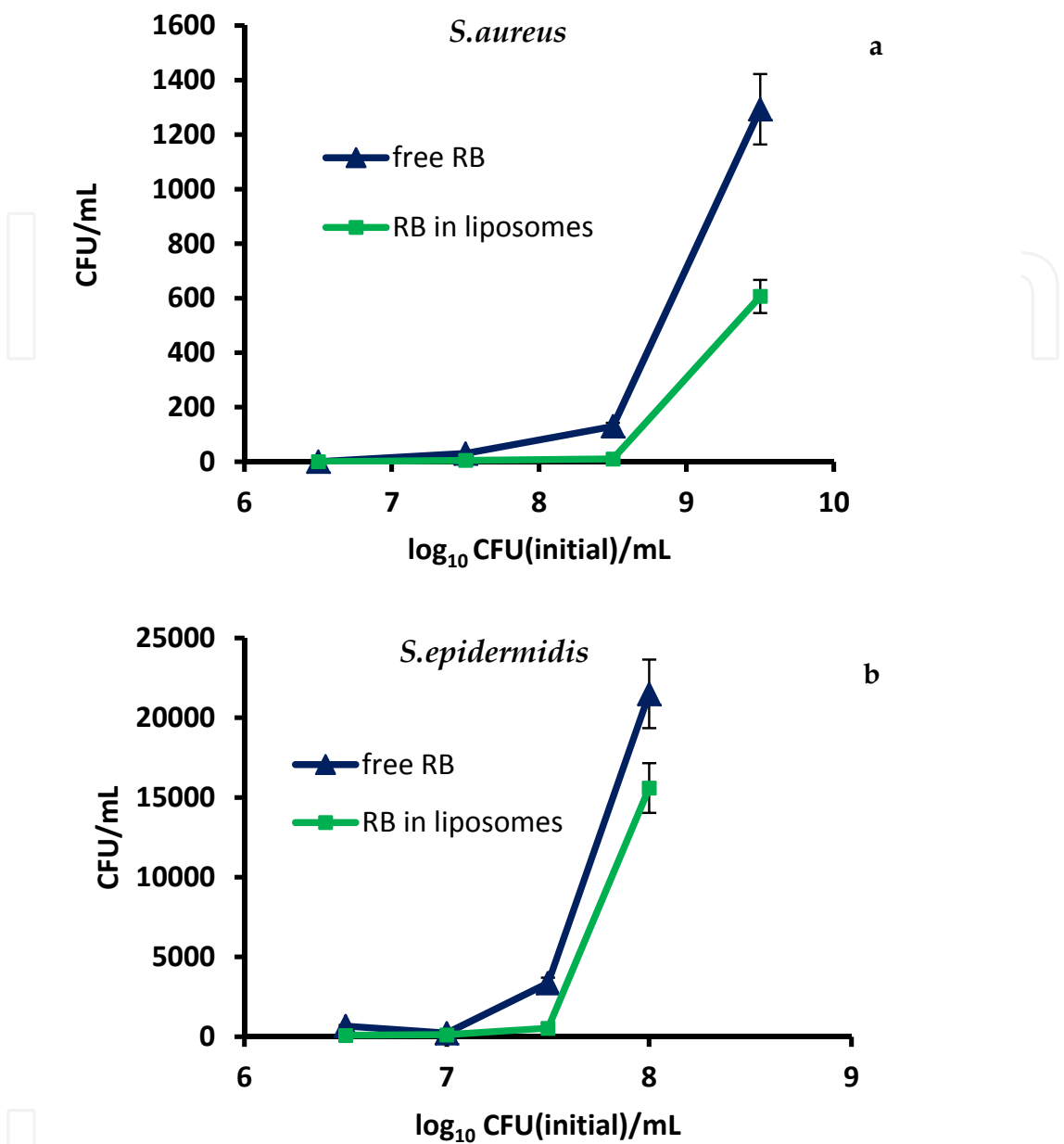


Fig. 10. Eradication of (a) *S. aureus* by 0.25 μ M and (b) *S. epidermidis* by 0.01 μ M Rose Bengal (RB) in a free form and encapsulated into EPC-olive oil liposomes under white light illumination at various initial bacteria concentrations presented in a logarithmic form.

Olive oil-containing liposomes with encapsulated Methylene Blue were tested against *S. epidermidis*. Bacterial sensitivity to this photosensitizer was much lower than to Rose Bengal in both free and liposomal forms. Thus, at the same initial bacterial concentration of $3 \cdot 10^6$ cells/mL, total eradication of *S. epidermidis* by liposomal Rose Bengal was achieved at 0.02 μ M (Fig. 9b), and by liposomal Methylene Blue only at a concentration of 62.5 μ M (Fig. 12). As to the general effect of free and liposomal Methylene Blue, it can be said that this photosensitizer exhibits the same trends as Rose Bengal. A liposome-encapsulated form was twice to three times more effective than the free form at all Methylene Blue concentrations (Fig. 12).

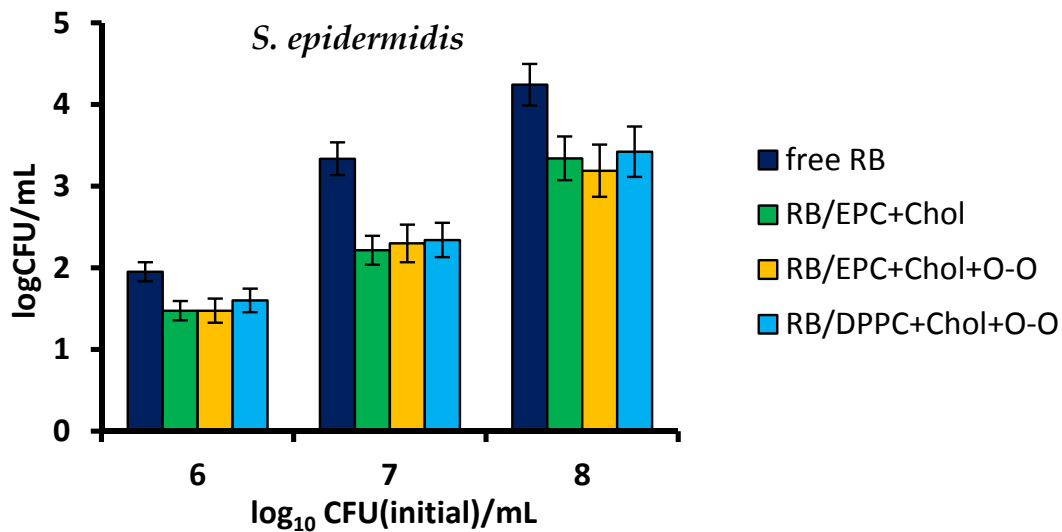


Fig. 11. Eradication of *S. epidermidis* under white light illumination by 0.01μM Rose Bengal (RB) in a free form and when encapsulated into liposomes with or without olive oil (O-O) and cholesterol (Chol) at various initial bacteria concentrations presented in a logarithmic form.

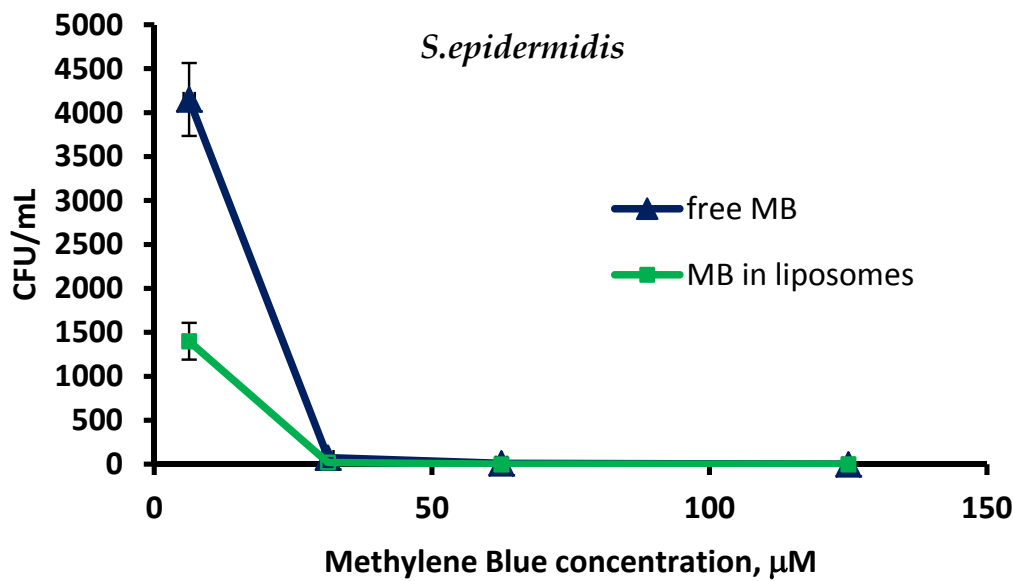


Fig. 12. Eradication of *S. epidermidis* by various concentrations of Methylene Blue in a free form and encapsulated into EPC–olive oil liposomes under white light illumination at initial bacteria concentration of 3·10⁶ cells/mL.

It is important to mention that in no case did olive oil incorporation into the membrane of liposomes with encapsulated photosensitizers cause any decrease in their antimicrobial activity.

5. Perspectives for application of olive oil-containing liposomes

Several types of drug delivery systems containing lipids for oral, intravenous or dermal administration are described in the literature (Wasan, 2007). One of them is an oil-in-water

emulsion, composed of isotropic mixtures of oil triacylglycerols, surfactant and one or more hydrophilic solvents. The typical particle size of such systems is between 100 and 300 nm (Constantinides, 1995). Another system, called a lipidic self-microemulsifying drug delivery system, represents transparent microemulsions with a particle size of 50-100 nm (Constantinides, 1995; Holm et al., 2003). The described emulsions and microemulsions were based on structural triacylglycerols or sunflower oil. Such systems were proven to appropriately deliver lipophilic drugs such as cyclosporine A, saquinavir, ritonavir and halofantrine (Charman et al., 1992; Holm et al., 2002). A soybean lecithin-based nanoemulsion enriched with triacylglycerols was used for efficient delivery of Amphotericin B (Filippin et al., 2008). An additional example represents solid lipid nanoparticles which were shown to not only deliver glucocorticoids, but also to enhance drug penetration into the skin (Schlupp et al., 2011). Colloid dispersions of solid triacylglycerol 140 nm-sized nanoparticles stabilized with poly(vinyl alcohol) were applied for delivery of the drugs diazepam and ubidecarenone (Rosenblat & Bunje, 2009). Soybean and olive oils were suggested as drug delivery vehicles for the steroids progesterone, estradiol and testosterone (Land et al., 2005). All of the above-mentioned examples illustrate successful use of lipid-based systems for delivery of hydrophobic drugs. However, they are all unsuitable for carrying hydrophilic components.

Liposomes are devoid of this serious disadvantage and are applicable for delivery of both hydrophobic and hydrophilic agents. In case of dermal application, lipid-based drug formulations exhibit enhanced abilities to penetrate into skin, improving the delivery process of active agents, thus enabling an increase in treatment efficiency in cases of skin infections and inflammations caused by bacterial invasion. Liposomes were shown to carry the encapsulated hydrophilic agents into the human stratum corneum and possibly into the deeper layers of the skin (Verma et al., 2003). Packaging of drugs into liposomes enables a more concentrated delivery, enhanced cytotoxicity, improved pharmacokinetic qualities, sustained release and prolonged action of active components.

In this chapter we considered only one type of antimicrobial agents delivered by olive oil-containing liposomes, but the list of active drugs can be continued and expanded. Incorporation of olive oil into the lipid bilayer increases the biocompatibility of liposomes and enriches them with a broad spectrum of natural bioactive compounds. Integration of olive oil into the liposome lipid bilayer enriches the liposome features by new properties. Such enriched liposomes can not only fulfill a passive role in drug delivery, but can also supply active components for post-treatment recovery of skin. It has been proven that daily treatment with olive oil lowered the risk of dermatitis (Kiechl-Kohlendorfer et al., 2008). Olive oil vitamins and antioxidants could help overcome skin damage caused by skin infection and by the active treatment itself. Olive oil-containing liposomes can thus be converted from passive excipients into active supporting means of drug delivery systems. Totally natural and biocompatible olive oil-containing liposomes carrying any of the antimicrobial agents can be administrated in ointments and creams for application on skin areas contaminated with bacteria.

6. Conclusions

Olive oil can be incorporated into the liposome phospholipid bilayer, composed of an egg phosphatidylcholine or a dipalmitoyl phosphatidylcholine bilayer. The photosensitizers Rose Bengal and Methylene Blue encapsulated in olive oil-containing liposomes showed

high efficiency in the eradication of Gram-positive *Staphylococcus aureus* and *Staphylococcus epidermidis* bacteria. The effectiveness of the antimicrobial agents was concentration-sensitive and depended on the initial concentration of the bacteria.

Application of olive oil-containing liposomes for drug delivery can change their perception as having a passive role of lipid-based excipients, converting them into a new generation of active and supporting drug carriers, supplying natural bioactive components for post-treatment recovery of skin.

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8. References

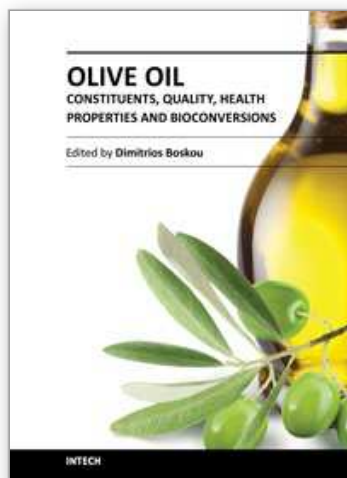
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Olive Oil - Constituents, Quality, Health Properties and Bioconversions

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The health-promoting effects attributed to olive oil, and the development of the olive oil industry have intensified the quest for new information, stimulating wide areas of research. This book is a source of recently accumulated information. It covers a broad range of topics from chemistry, technology, and quality assessment, to bioavailability and function of important molecules, recovery of bioactive compounds, preparation of olive oil-based functional products, and identification of novel pharmacological targets for the prevention and treatment of certain diseases.

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