Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochimica et Biophysica Acta 1611 (2003) 187–196
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.bba-direct.com

A spin labelling study of immunomodulating peptidoglycan monomer and adamantyltripeptides entrapped into liposomes

Ruža Frkanec[a], Vesna Noethig-Laslo[b,*], Branka Vranešić[a], Krunoslav Mirosavljević[b], Jelka Tomašić[a]

[a] *Institute of Immunology Inc., P.O. Box 266, 10001 Zagreb, Croatia*
[b] *Rudjer Bošković Institute, Bijenička c. 54, 10002 Zagreb, Croatia*

Received 19 November 2002; received in revised form 31 January 2003; accepted 11 February 2003

Abstract

The interaction of immunostimulating compounds, the peptidoglycan monomer (PGM) and structurally related adamantyltripeptides (AdTP1 and AdTP2), respectively, with phospholipids in liposomal bilayers were investigated by electron paramagnetic resonance spectroscopy. (1) The fatty acids bearing the nitroxide spin label at different positions along the acyl chain were used to investigate the interaction of tested compounds with negatively charged multilamellar liposomes. Electron spin resonance (ESR) spectra were studied at 290 and 310 K. The entrapment of the adamantyltripeptides affected the motional properties of all spin labelled lipids, while the entrapment of PGM had no effect. (2) Spin labelled PGM was prepared and the novel compound bearing the spin label attached via the amino group of diaminopimelic acid was chromatographically purified and chemically characterized. The rotational correlation time of the spin labelled molecule dissolved in buffer at pH 7.4 was studied as a function of temperature. The conformational change was observed above 300 K. The same effect was observed with the spin labelled PGM incorporated into liposomes. Such effect was not observed when the spin labelled PGM was studied at alkaline pH, probably due to the hydrolysis of PGM molecule. The study of possible interaction with liposomal membrane is relevant to the use of tested compounds incorporated into liposomes, as adjuvants in vivo.

Keywords: Spin labelling; Peptidoglycan monomer; Adamantyltripeptide; Liposome; Phospholipid

1. Introduction

Adjuvants or immunostimulating compounds are used to enhance the immune response of the host to an antigen. Such compounds are particularly required for stimulating the immune response to weak antigens and vaccines. The search for new adjuvants has been very intensive in the last decades. Among others, a series of compounds comprising the elements of the structure of bacterial peptidoglycans have been studied and the results so far indicate that several of such compounds could be considered as potential new adjuvants. Peptidoglycans are ubiquitous constituents of bacterial cell walls responsible for the physical integrity of bacteria. They are composed of glycan chains, which are built of β-1,4-linked *N*-acetylglucosamine and *N*-acetylmuramic acid residues and peptide units that consist of alternating L- and D-amino acids. Peptidoglycan fragments exhibit various biological activities that depend upon the size and composition of the peptidoglycan fragments [1]. One of the most prominent and well-documented activities is the effect of peptidoglycans on the mammalian immune system. Low molecular weight peptidoglycan fragments, either obtained from natural sources or prepared synthetically, are mostly devoid of the toxic properties characteristic for large peptidoglycans, but still retain marked immunomodulating activity [2–6].

Abbreviations: PGM, peptidoglycan monomer, GlcNAc-MurNAc-L-Ala-D-isoGln-mesoDAP(ωNH$_2$)-D-Ala-D-Ala; AdTP1, D-(adamant-2-yl)-Gly-L-Ala-D-isoGln; AdTP2, L-(adamant-2-yl)-Gly-L-Ala-D-isoGln; ESR, electron spin resonance; Egg-PC, L-α-phosphatidylcholine, type XI-E: from fresh egg yolk; CHL, cholesterol from porcine liver; DCPh, dicetyl phosphate; *n*-doxyl SA, *n*-doxyl-stearic acids (*n*=5, 7, 16); DCC, *N*,*N*′-dicyclohexyl-carbodiimide; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; DMF, dimethylformamide; TFA, trifluoroacetic acid

* Corresponding author. Tel.: +385-1-4561-136; fax: +385-1-4680-245.
E-mail address: laslo@faust.irb.hr (V. Noethig-Laslo).

0005-2736/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0005-2736(03)00054-3

Our studies concern peptidoglycan monomer, GlcNAc-MurNAc-L-Ala-D-isoGln-mesoDAP(ωNH$_2$)-D-Ala-D-Ala (PGM), the natural compound originating from the *Brevibacterium divaricatum* peptidoglycan [7] (Fig. 1A) and synthetic adamantyltripeptides (Fig. 1B). PGM is a water-soluble, non-toxic and non-pyrogenic substance and its chemical structure is completely defined [8,9].

Synthetic adamantyltripeptides, D-(adamant-2-yl)-Gly-L-Ala-D-isoGln (AdTP1) and L-(adamant-2-yl)-Gly-L-Ala-D-isoGln (AdTP2) [10] comprise adamantyl moiety linked to the dipeptide L-Ala-D-isoGln, which is the structural element of the natural peptidoglycans. We have recently shown in a mice model, using the ovalbumin as an antigen, that PGM and AdTP2 possess adjuvant properties [11,12]. We have also shown earlier that these compounds undergo rapid biotransformation by the action of hydrolytic enzymes and/or excretion in vivo [13–15]. It could be assumed that a hydrolysis and excretion would terminate the immunostimulating activity [16].

Therefore, it was of interest to find the appropriate formulation, like incorporation of studied compounds into liposomes with the aim to slow down their hydrolytic degradation and thus achieve the prolonged action and effect on the immune system. Based on earlier reports [17–19] concerning the fate and biodistribution of liposomes in the mammalian organism, we have chosen negatively charged, multilamellar liposomes as carriers for our compounds.

In order to show how the examined peptides incorporated into liposomes affect membrane properties, electron spin resonance (ESR), a spin labelling technique, was used. The method is based on a dynamic sensitivity of the nitroxide label to the time-scale of the rotational motions of lipids and proteins in biological membranes [20–22]. Spin labelling was successfully applied in the study of interactions of immunologically active lipopeptides with membranes [23]. Influence of the liposome composition on the bilayer fluidity and adsorption of the liposomes into the skin was investigated as well, in order to resolve molecular mechanisms by which penetration of liposomal drugs were facilitated [24].

Immunostimulating peptides studied in this work are soluble in water and at pH 7.4 are in the ionized form. Therefore, one can envisage charge–charge interactions between the peptides examined and liposomes. Recently, charge–charge interactions between liposomes and spermine [25], or glycine [26] were shown by spin labelled fatty acids incorporated into the liposomes.

In the present work, we have studied ESR spectra of peptidoglycan monomer (PGM) and adamantyltripeptides incorporated into liposomes in order to get information of possible interaction of these compounds with lipids. Also,

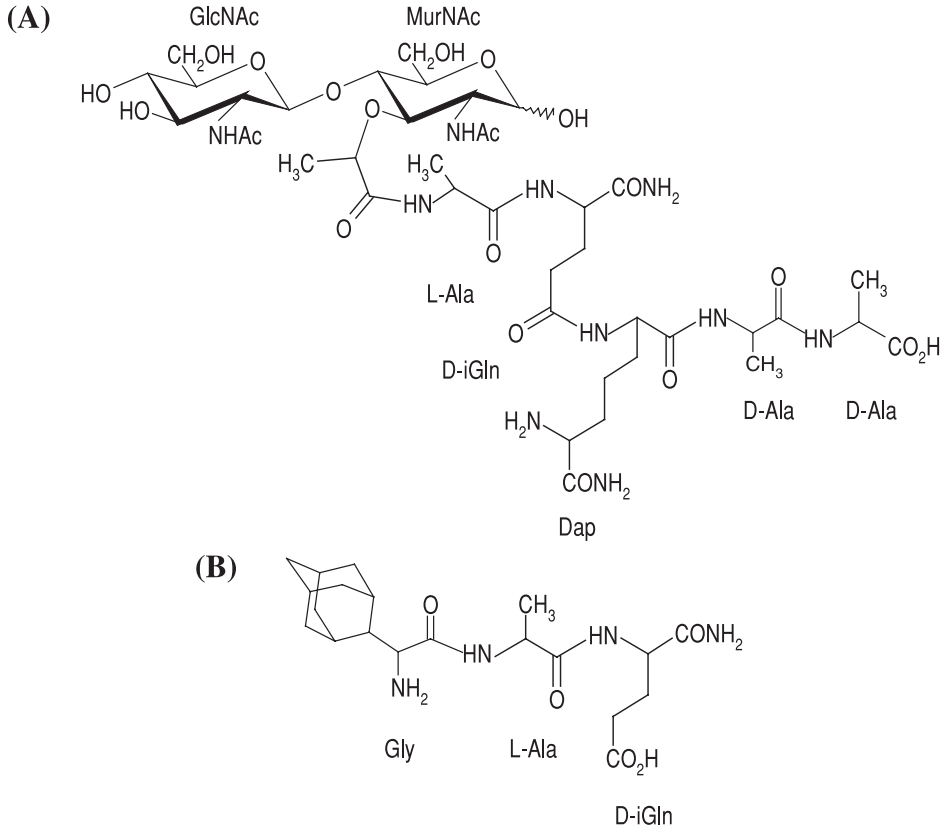

Fig. 1. Structural formula of the examined compounds: (A) peptidoglycan monomer (PGM), (B) adamantyltripeptides (AdTP1 and AdTP2).

the PGM was spin labelled and incorporated into liposomes. ESR spectra of spin labelled PGM were studied as a function of temperature.

2. Materials and methods

L-α-Phosphatidylcholine, type XI-E: from fresh egg yolk (egg-PC), cholesterol from porcine liver (CHL), dicetyl phosphate (DCPh), the spin labelled fatty acids, *n*-doxyl-stearic acids (*n*-doxyl SA) (*n* = 5, 7, 16), *N*,*N*′ -dicyclohexyl-carbodiimide (DCC) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and 3-carboxy-proxyl (3-proxyl) was synthesized by the procedure described in Ref. [27].

2.1. Peptidoglycan and adamantyltripeptides

GlcNAc-MurNAc-L-Ala-D-isoGln-mesoDAP(ωNH$_2$)-D-Ala-D-Ala, (PGM), was prepared in PLIVA, Chemical and Pharmaceutical Works (Zagreb, Croatia), according to the previously described procedure [7]. Two diastereoisomers AdTP2 and AdTP1 were prepared as described in Ref. [10]. Acetonitrile and trifluoroacetic acid (TFA) were of HPLC grade (Merck, Darmstadt, Germany). A daily supply of water was obtained from Simplicity-Personal ultra pure water system (Millipore, Bedford, MA, USA). All other chemicals used for buffers were of analytical grade and purchased from Sigma.

Column chromatography was performed on Sephadex LH-20, (Pharmacia, Uppsala, Sweden).

Thin-layer chromatography (TLC) was performed on a Silica Gel 60 (Merck) and detection was performed with chlorine/*o*-tolidine reagent or with J_2. Two solvent systems: (A) *n*-BuOH/HOAc/H_2O (6:1.5:2.5) and (B) EtOAc/*n*-PrOH/H_2O (1:2:2) were used.

Homogeneity and retention time of the tested compounds were determined by HPLC on a Waters 600E HPLC System utilizing an analytical Merck Lichrosorb RP-18 column (4 × 244 mm) and a Merck guard column LiChrospher 100 RP-18 under gradient conditions [28]. The flow rate was 1 ml/min at room temperature, UV detection at 214 nm.

All analyses were performed at ambient temperature. The gradient solvent systems used were composed of acetonitrile containing 0.035% TFA and water containing 0.05% TFA. Two gradient systems were used for the tested compounds. Each system contained a different percentage of acetonitrile and the amount of acetonitrile was changed at the indicated running times. For system A with a running time 35 min, the percentage of acetonitrile at 0, 30 and 35 min was 3, 20 and 3, respectively. For system B, with a running time of 20 min, the percentage of acetonitrile at 0, 15 and 20 min was 10, 30 and 10, respectively.

System A was used for analyses of the free spin label and for the spin labelled PGM; system B was used for analyses of adamantyltripeptides.

2.2. Preparation of liposomes

Egg-PC 28.4 mg (0.37 mmol); CHL 9.8 mg (0.25 mmol) and DCPh 2.7 mg (0.05 mmol), giving a molar ratio of 7:5:1, were dissolved in 4 ml of $CHCl_3$. After rotary evaporation of the solvent, the remaining lipid film was dried in vacuum for an hour and then dispersed by vigorous hand shaking in 2.5 ml phosphate buffered saline (PBS). PGM or adamantyltripeptides were incorporated into liposomes as follows: 1 ml of the ethanol solution (5 mM) of each compounds, respectively, was added to the lipids in the chloroform solution and the procedure confirmed as described above for empty liposomes.

2.3. Spin labelling of liposomes

Liposomes were spin labelled with *n*-doxyl SA (*n* = 5, 7, 16) by a thin film of the spin labels (1% with respect to the phospholipids of liposomes) formed after evaporation of the organic solvent under reduced pressure [17]. Eight liposome spin labelling preparations were studied.

2.4. Preparation of spin labelled peptidoglycan monomer

2.4.1. Preparation of symmetrical anhydride of 3-carboxy-proxyl

Activation of carboxyl group of 3-proxyl was achieved by condensation of acid (51.4 mg, 0.29 mmol) in the presence of DCC (65.0 mg, 0.32 mmol). The urea derivative was removed by filtration and, upon evaporation of filtrate, the crude anhydride was used in the next step without further purification.

Spin labelled PGM was prepared by condensation of unprotected PGM (35.9 mg, 0.04 mmol) with an excess of symmetrical anhydride of 3-proxyl acid in the presence of 30 μl triethylamine in 2 ml dimethylformamide. The reaction was monitored by TLC in solvent system B. After removal of DMF in vacuum, the residue was dissolved in water and dicyclourea was removed by filtration. Crude product was applied to a column of Sephadex LH-20 (2.5 × 90 cm) and eluted with water/ethanol (1:1 v/v). Absorbance of the fraction was measured at 230 nm and the fractions corresponding to spin labelled PGM were pooled and evaporated to dryness. Final purification was achieved by repeating the chromatography on Sephadex LH-20 (2.5 × 90 cm) and was eluted with water/ethanol (1:1 v/v).

The purity of the obtained spin labelled PGM was checked by high-pressure liquid chromatography (HPLC) and its composition confirmed by total hydrolysis.

2.5. ESR measurements

ESR measurements were done on Varian E-9 spectrometer (10 GHz) equipped with a Bruker variable temperature

control unit. The spectra were recorded with digital acquisition, EW-ESR ware [29]. Sample capillaries were inserted into the standard 4 mm diameter ESR quartz tubes and centred in a TE_{102} ESR cavity. At each temperature, the samples were left for about 10 min for temperature equilibration.

ESR spectra of doxyl stearic acids in liposomes reflect motional properties of the lipid bilayer. One important motional component of the lipid bilayer is "wobbling" of the long molecular axis about a direction near perpendicular to the bilayer surface. This motional effect is measured by the ESR experimental parameters: a maximal hyperfine splitting A_{max} (mT), and a minimal hyperfine splitting A_{min} (mT), respectively (Figs. 2a and 3a), from which the order parameter S [30–32] was calculated by the Eq. (1):

$$S = 3(A_{max} - A_{min})/(A_{max} + 2A_{min}) \times 0.5396 \quad (1)$$

The line widths ($\Delta H/mT$) of the ESR spectra of the spin labelled PGM were fitted with Simfonia program (Bruker)

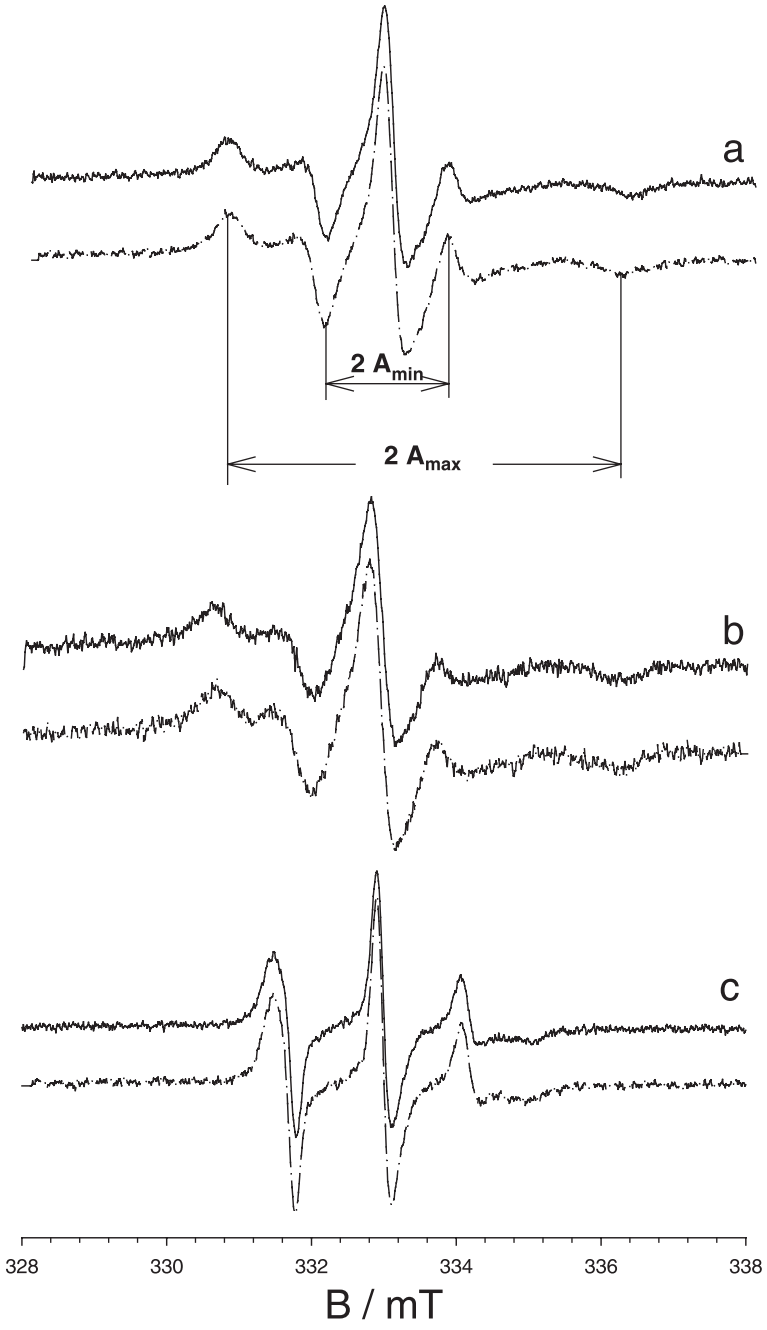

Fig. 2. ESR spectra, taken at 290 K, of the spin labelled liposomes with n-doxyl SA: a—$n=5$; b—$n=7$; c—$n=16$, prepared with the entrapped AdTP1 (full lines) and empty liposomes (dotted lines).

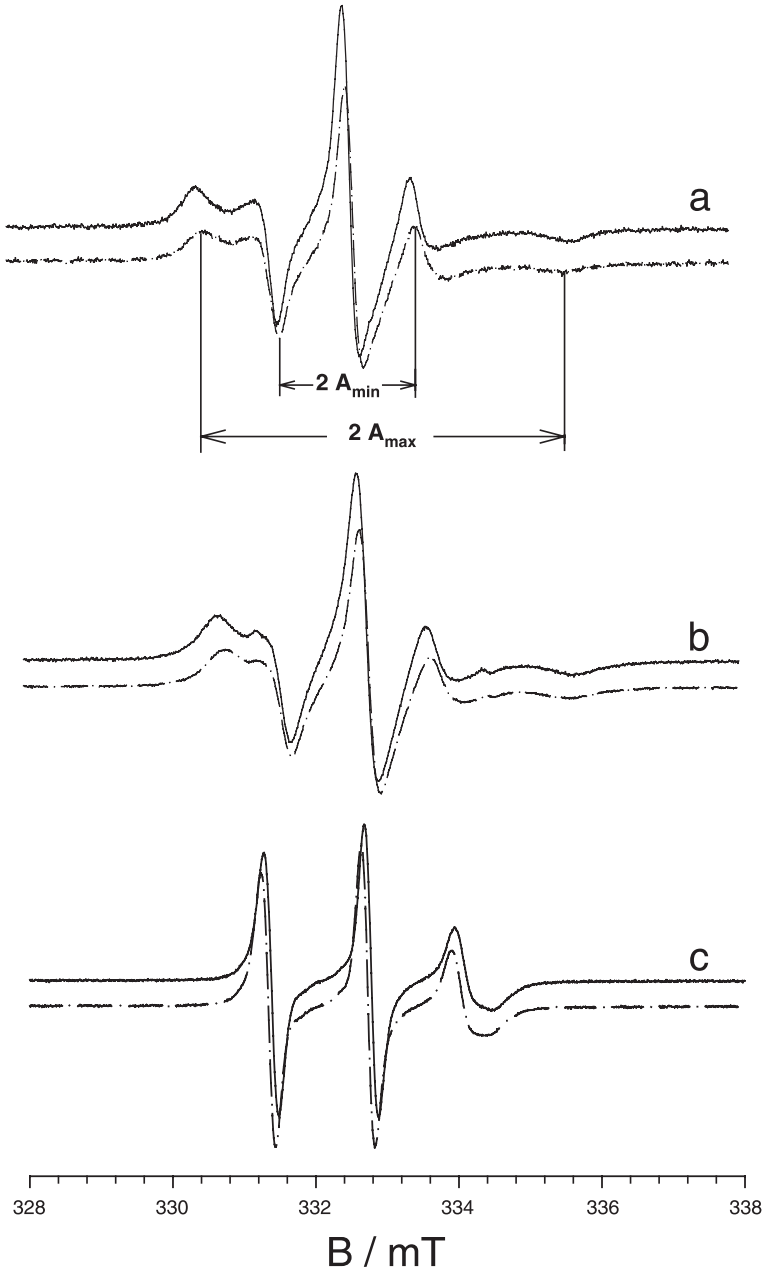

Fig. 3. The comparison of ESR spectra, recorded at 310 K, of the spin labelled liposomes (*n*-doxyl SA: a—$n=5$; b—$n=7$; c—$n=16$) with entrapped AdTP2 (full lines) with the empty liposomes (dotted lines).

by Eq. (2) describing the line widths dependence on the magnetic moment of the nitrogen nucleus [33,34]:

$$\Delta H = A + Bm_N + Cm_N^2 \quad (2)$$

where $A=\Delta H(0)=\Delta v(0)h/g\beta$ is the central line width. From the line shapes of the ESR spectra fitted with parameters B and C, a rotational correlation time, τ_2, the parameter of the motion of the spin labelled PGM was calculated by Eq. (3) [34]:

$$\tau_2 = -1.22 \times 10^{-9} B \quad (3)$$

the parameter B being the only one varied by temperature changes. It might be supposed that the spin labelled PGM molecule describes Brownian motion. From the Stokes–Einstein relation, the rotational correlation time of the PGM molecule can be approximated by Eq. (4) [33]:

$$\tau_2 = 1/kV_{\text{eff}}(\eta/T) \quad (4)$$

where V_{eff} is the effective volume of the PGM molecule, η the viscosity of the solvent, k the Boltzmann constant and T temperature. If the molecule of the PGM retains one conformation in the whole temperature interval examined, τ_2 as a function of $1/T$ should be a straight line, with the slope proportional to the effective volume (V_{eff}) of the spin labelled PGM. Changes of the slope of the

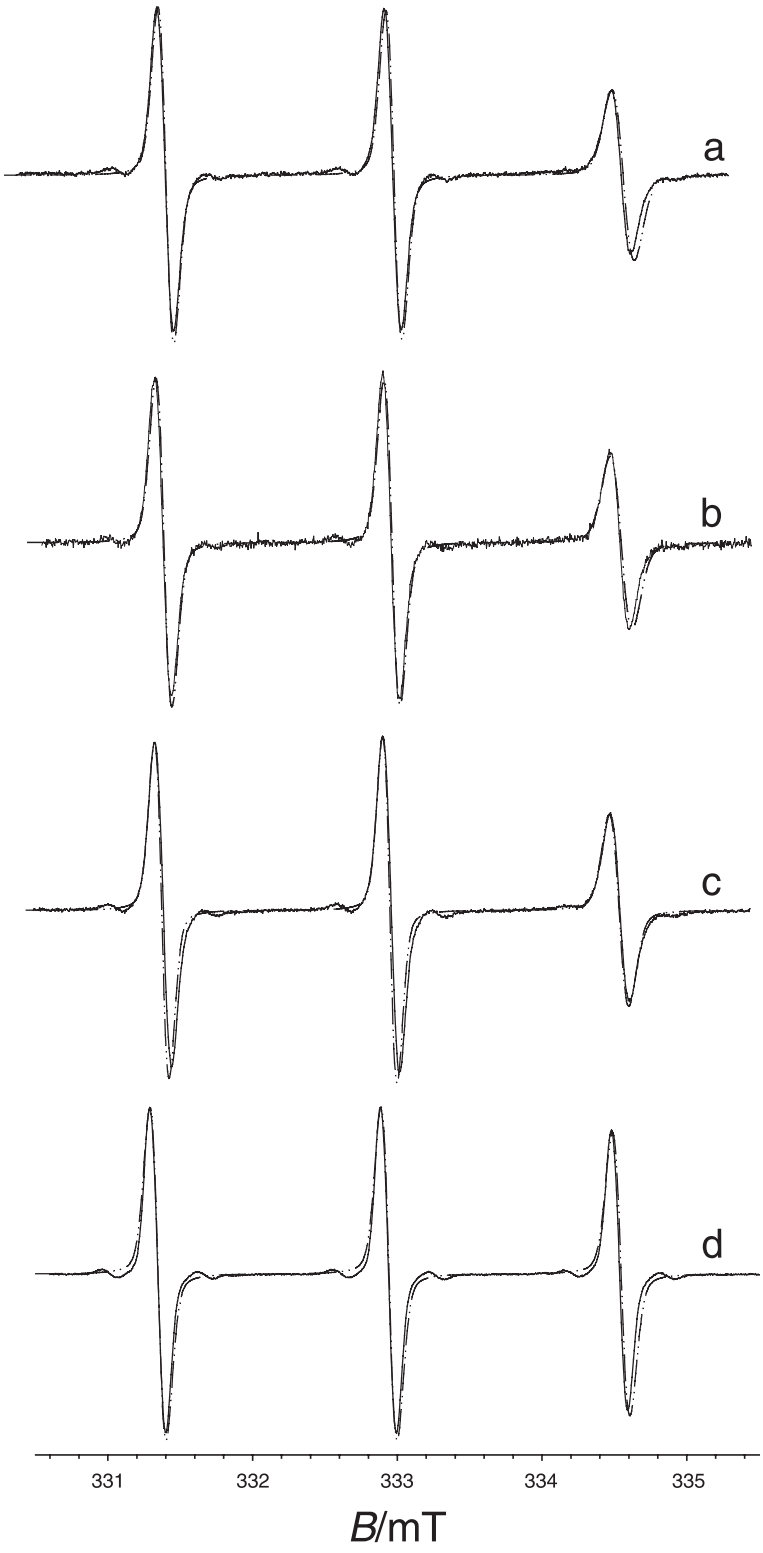

Fig. 4. ESR spectra of the spin labelled PGM entrapped in the liposomes (spectrum a, $\tau_2 = 367$ ps), in PBS buffer at pH 7.4 (spectrum b, $\tau_2 = 349$ ps), in the phosphate buffer at pH 10.0, (spectrum c, $\tau_2 = 274$ ps) and of the free spin label (3-carboxy-proxyl) at pH 7.4 in PBS buffer (spectrum d, $\tau_2 = 76.4$ ps). The spectra were taken at 290 K. The line shapes of the experimental spectra (full lines) were fitted with the Simfonia program (Bruker) (dotted lines) from which the rotational correlation time, τ_2, was calculated [33].

straight line would suggest a change of the peptide conformation.

3. Results

3.1. Interactions between peptides examined and the multilamellar liposomes

The compounds examined are well soluble in water but not in the nonpolar solvents. At pH 7.4 these compounds are presumably present in an ionized form while liposome of chosen composition (egg-PC/CHL/DCPh, 7:5:1) bears the negative charge. So charge–charge interactions between peptides examined and the liposome surface may be envisaged. Hydrophobic interaction might be expected for the two adamantyltripeptides, since adamantan moiety has the lipophilic character, which could facilitate the adsorption of the peptides on the surface of the liposomes.

In order to check a possible adsorption of the tested compounds entrapped into multilamellar liposomes, spin labelled liposomes (with *n*-doxyl SA; $n=5$, 7, 16) were prepared in the presence and in the absence of two adamantyltripeptides examined, AdTP1 and AdTP2, respectively, as well as in the presence of PGM. ESR spectra of the spin labelled liposomes were studied at two different temperatures, 290 K (17 °C) and 310 K (37 °C).

In Fig. 2, ESR spectra, taken at 290 K, of the spin labelled liposomes (*n*-doxyl SA: a—$n=5$; b—$n=7$; c—$n=16$) prepared with the entrapped AdTP1 (full lines) are compared with the empty liposomes (dotted lines). An increase of $2A_{max}$, with a concomitant decrease in $2A_{min}$ was determined in the spectra of all the three spin labels in the liposomes with entrapped AdTP1 as compared to the spectra of the empty liposomes. This was reflected as an increased order parameter *S* calculated from the spectra.

In Fig. 3, ESR spectra, recorded at 310 K, of the spin labelled liposomes (*n*-doxyl SA: a—$n=5$; b—$n=7$; c—$n=16$) with entrapped AdTP2 (full lines) are compared with the empty liposomes (dotted lines). As in the case of AdTP1, an increase in $2A_{max}$ with concomitant decrease in $2A_{min}$ was detected.

No change in the motional properties of all the spin labels was observed when PGM was incorporated into liposomes.

In Table 1, the order parameter *S*, calculated from the ESR spectra taken at 37 °C (the average values of the three different liposome preparations and of the eight spin labelling) with entrapped peptides are compared to the empty liposomes. An increase in the order parameter *S* was calculated for all the spin labelled fatty acids when adamantyltripeptides were entrapped in the liposomes, while there was no change in the order parameter *S* in the case of PGM entrapped into liposomes.

3.2. Spin labelled PGM

In order to study conformational properties of PGM and to check if there are some effects of the liposomes on the conformation of the entrapped PGM, the glycopeptide molecule was spin labelled at the gamma-NH_2 group of the mesodiaminopimelic acid.

In Fig. 4, ESR spectra of the spin labelled PGM entrapped in the liposomes (spectrum a), in PBS buffer at pH 7.4 (spectrum b), in the phosphate buffer at pH 10.0, (spectrum c) and of the free spin label (3-proxyl) at pH 7.4 in PBS buffer (spectrum d) are shown. The line shapes of the spectra (full lines) were fitted with the Simfonia program (Bruker) (dotted lines) from which the rotational correlation

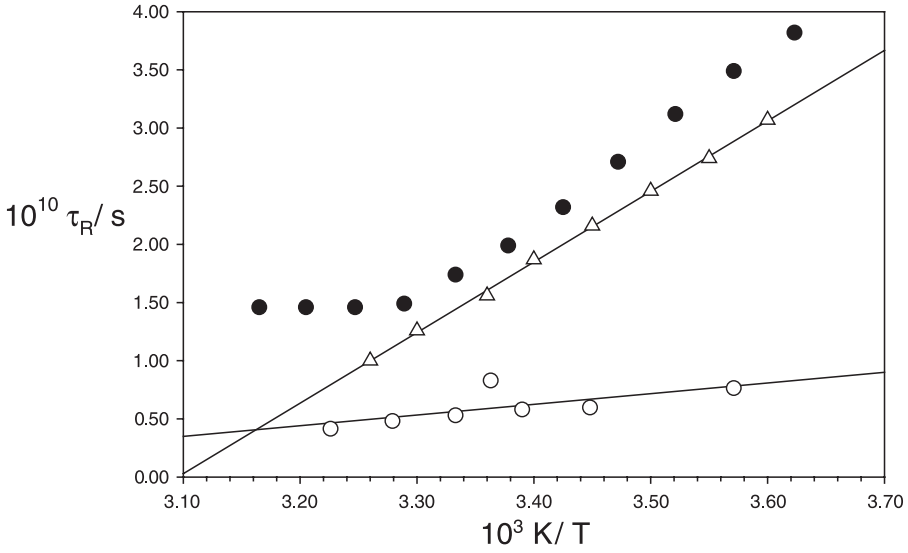

Fig. 5. The rotational correlation time, τ_2, of the spin labelled PGM, as a function of $1/T$, in PBS buffer at pH 7.4 (black circles), in PBS buffer at pH 10.0, (white triangles), as well as the τ_2 of the free spin label (white circles).

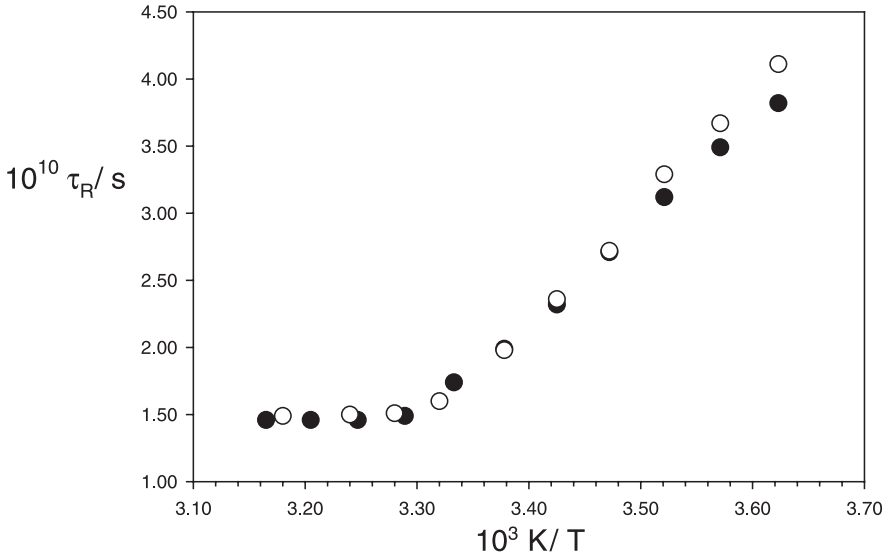

Fig. 6. Dependence τ_2 on $1/T$ of the spin labelled PGM in buffer at pH = 7.4 (black circles), and incorporated into liposomes (white circles).

time, τ_2, was calculated [33]. It was supposed that the rotational correlation time, τ_2, well described the motion of the whole spin labelled glycopeptide molecule.

The spectra of the spin labelled PGM were studied as a function of pH at: 4.2 $\tau_2 = 320$ ps; 5.84 $\tau_2 = 319$ ps; 7.04 $\tau_2 = 320$ ps; 8.1 $\tau_2 = 314$ ps; 10.0 $\tau_2 = 307$ ps, in 0.1 M phosphate buffer, at 278 K. The values of τ_2 were obtained by the spectral simulations. Up to pH 8.1 the rotational correlation time of the spin labelled PGM remained unchanged within the experimental error. An increase of the motional freedom of the spin labelled PGM was detected only at pH 10.0 (under conditions of the alkaline hydrolysis of PGM [7,35]).

In Fig. 5, the rotational correlation time, τ_2, of the spin labelled PGM, as a function of $1/T$, in PBS buffer at pH 7.4 (black circles), in phosphate buffer at pH 10.0, (white triangles), as well as the τ_2 of the free spin label (white circles) is displayed.

If the molecule of the spin labelled PGM retains one conformation in the whole temperature interval examined, the straight line of τ_2 on $1/T$ should be obtained, with the slope proportional to the effective volume of the spin labelled molecule.

The temperature dependence of τ_2, of the spin labelled PGM at pH 7.4 (black circles) cannot be approximated by a straight line. Above 303 K, the spin label motion does not reflect the increase of the temperature any more. This suggests the increase of the constraint on the spin label motion above 303 K by some part of the PGM molecule, due to a conformation change. The straight line of τ_2 on $1/T$ was obtained for the free spin label (white circles), with the smaller slope of the straight line suggesting a smaller effective volume of the solvated free spin label than the spin labelled PGM. At pH 10.0, the straight line of τ_2 on $1/T$ was also obtained (white triangles) suggesting smaller effective volume of the spin labelled peptide formed by alkaline hydrolyses from the whole spin labelled PGM molecule and no change of the conformation.

In Fig. 6, dependence of τ_2 on $1/T$ of the spin labelled PGM in buffer at pH = 7.4 (black circles), and incorporated into liposomes (white circles) is displayed. Incorporation into the liposomes has only a small effect on the slope of the function τ_2 on $1/T$, but does not affect the temperature of the intersection of the two straight lines, suggesting that liposomes have no effect on the temperature dependent conformation change of the PGM molecule.

4. Discussion

In several studies reported earlier, different compounds comprising the elements of peptidoglycan structure were incorporated into liposomes. Most of these studies concern the synthetically prepared derivatives of muramyldipeptide [36–38]. Liposomal preparation of such compounds in some cases exhibited markedly better biological activity [39]. The liposomal preparation of muramyltripeptide phosphatidylethanolamine (L-MTP-PE) has been applied successfully in cancer therapy and is undergoing clinical trials [40,41]. Our former study concerned the incorporation of PGM into negatively charged multilamellar liposomes and the amount of the entrapped material was determined using C-14 labelled PGM [42]. The amount of the entrapped peptidoglycans or derivatives of muramyldipeptide varied with regard to their lipophilicity and the type of liposomes. However, to the best of our knowledge, the interaction of the entrapped material with the lipid membrane using ESR has not been studied or reported so far. Such studies could be carried out using the spin labels either on the lipid portions of liposomal membrane, or on the compound

incorporated into liposomes. It should also be pointed out that spin labelling of peptidoglycans or structurally related compounds have not been reported so far.

In this work we have, therefore, studied the possible interactions of three structurally related, biologically active compounds, namely PGM, AdTP1 and AdTP2, respectively, with the lipids in negatively charged multilamellar liposomes.

Our choice of large multilamellar, negatively charged liposomes could be explained as follows. According to the previously published reports [19], the negative charge and the larger size of liposomes favours the interaction with the reticuloendothelial systems. Negative charge is recognised by the receptors found on immunocompetent cells, including macrophages. Upon subcutaneous administration used in our model systems in mice, large multilamellar liposomes are expected to remain trapped for a prolonged period at the injection site and serve as a drug depot. Our recent preliminary studies of immunostimulating activity of PGM incorporated in negatively charged multilamellar liposomes demonstrated the efficacy of chosen liposomal preparations (unpublished results).

In the first part of the study, spin labelled fatty acids (*n*-doxyl SA) with paramagnetic nitroxide moiety positioned at different carbon atoms were used for liposome preparations and PGM, AdTP1 and AdTP2 incorporated in the respective preparations. ESR spectra of spin labelled multilamellar liposomes were studied at two temperatures: 290 and 310 K. It was observed that the entrapment of adamantyltripeptides, both AdTP1 as well as AdTP2, affected motional properties of all lipid spin labels. An increase of the order parameter *S* was determined with all the spin labels in the liposomes prepared with AdTP1 or AdTP2 as compared to the empty liposomes (Table 1). This effect of adamantyltripeptides was more pronounced when the spin labelling and ESR spectra were taken at 310 K.

A nature of a possible ‘‘binding’’ of the adamantyltripeptides to the surface of the liposomes examined in this work could be discussed. The presence of the adamantyltripeptides influenced even the motional properties of 16-doxyl stearic acid placed in the hydrophobic core of the liposomes, although the peptides were not soluble in the organic solvents. This fact may be explained if we suppose that ‘‘binding’’ of the adamantyltripeptides to the liposome surface could induce conformation changes of the choline groups of the phospholipids. The other possibility would be that adamantyltripeptides induced changes in the lipid domains of the liposomes [43]. ‘‘Binding’’ of peptides should also depend on the ionic strength of the buffer. It is of interest to note that both adamantyltripeptide diastereoisomers showed the same ‘‘binding’’ to the liposome surface.

Table 1
Order parameter (*S*) of ESR spectra of the spin labelled fatty acids, taken at 310 K

Liposomes	5-doxyl-SA[a]	7-doxyl-SA[a]	16-doxyl-SA[a]
Empty liposomes	0.582 ± 0.05	0.531 ± 0.08	0.134 ± 0.08
Liposomes + PGM	0.590 ± 0.05	0.537 ± 0.08	0.143 ± 0.08
Liposomes + AdTP1	0.620 ± 0.05	0.560 ± 0.08	0.200 ± 0.08
Liposomes + AdTP2	0.619 ± 0.05	0.565 ± 0.08	0.195 ± 0.08

[a] SA—stearic acid.

Recently, similar effect of the amino acid glycine was observed on the spin labelled fatty acids in the liposomes of similar composition [26]. The effect may be of the similar nature as we observed for the two adamantyltripeptides. On the other hand, PGM entrapped in examined liposomal preparations had no effect on motional properties of lipid spin label regardless of the position of the spin label in the fatty acid chain. It could be assumed that PGM remains entrapped between lipid bilayers but has no direct interaction with the lipids.

We have therefore attempted another approach and prepared spin labelled PGM molecule. The synthesis was accomplished via anhydride method as described earlier for two PGM derivatives [43] using unprotected PGM and taking advantage of the available amino group at diaminopimelic acid. ESR spectra of the spin labelled molecule in buffer were studied first at different temperatures. PGM is a small glycopeptide, and thus, one can approximate the motion of the spin labelled molecule with unlabelled molecule. The rotational correlation time of the spin labelled PGM has to be linear function of the $1/T$ if there was no change in the glycopeptide conformation. Following this reasoning, we have shown that PGM underwent conformational change above 300 K. The same effect was observed when spin labelled PGM was entrapped into liposomes; again indicating that there was no interaction with the lipids, as demonstrated in the experiments with liposomes comprising spin labelled fatty acids.

Another experiment with spin labelled PGM was carried out at pH 10. It was shown previously that PGM is unstable at alkaline conditions and was consequently hydrolysed into disaccharide and lactylpentapeptide portions [7,34]. In the case of spin labelled PGM at pH 10, only the peptide portion retained the spin label. ESR study of such preparations demonstrated that there was no conformational change at 300 K. This finding indicates that the disaccharide part of the integral PGM molecule is essential for the conformational change at higher temperature. This fact could be relevant for biological activity of PGM in mammals with bodily temperatures above 310 K since the right conformation is the necessary requirement for reactions with cell receptors.

In conclusion, in this study, we have presented the data indicating that adamantyltripeptides interact with lipids in negatively charged multilamellar liposomes, while PGM did not directly interact with such lipids. Also, using spin labelled PGM, we have observed the conformational change in the glycopeptide molecule above 303 K. The conformational change was observed only with the complete molecule and not with the lactylpentapeptide portion generated in alkaline media.

Acknowledgements

We thank the Ministry of Science and Technology of Croatia for its financial support and Mrs. R. Jožinec for technical assistance in HPLC analyses.

References

[1] P.H. Seidl, K.H. Schleifer (Eds.), Biological Properties of Peptidoglycans, W. de Gruyter, Berlin, 1986.
[2] F. Ellouz, A. Adam, R. Ciorbaru, E. Lederer, Biochem. Biophys. Res. Commun. 59 (1974) 1317–1325.
[3] I. Azuma, Vaccine 10 (1992) 1000–1006.
[4] D.E.S. Stewart-Tull, Prog. Drug Res. 32 (1988) 305–328.
[5] U. Urleb, A. Krbavčič, M. Sollner, D. Kikelj, S. Pečar, Arch. Pharm. (Weinh.) 328 (1995) 113–117.
[6] D. Kikelj, S. Pečar, V. Kotnik, A. Štalc, B. Wraber-Herzog, S. Simičič, A. Ihan, L. Klamfer, L. Povšič, R. Grahek, E. Suhadolc, M. Hočevar, H. Honig, R. Rogi-Kohlenprath, J. Med. Chem. 41 (1998) 530–539.
[7] D. Keglevič, B. Ladešić, J. Tomašić, Z. Valinger, R. Naumski, Biochim. Biophys. Acta 585 (1979) 273–281.
[8] J. Tomašić, Lj. Sesartić, S.A. Martin, Z. Valinger, B. Ladešić, J. Chromatogr. 440 (1988) 405–414.
[9] H. Matter, L. Szilagyi, P. Forgo, Ž. Marinić, B. Klaić, J. Am. Chem. Soc. 119 (1997) 2212–2223.
[10] B. Vranešić, J. Tomašić, S. Smerdel, D. Kantoci, F. Benedetti, Helv. Chim. Acta 76 (1993) 1752–1758.
[11] J. Tomašić, I. Hanzl-Dujmović, B. Špoljar, B. Vranešić, M. Šantak, A. Jovičić, Vaccine 18 (2000) 1236–1243.
[12] B. Halassy Špoljar, T. Čimbora, I. Hanzl-Dujmović, B. Dojnović, A. Sabioncello, M. Krstanović, J. Tomašić, Vaccine 20 (2002) 3543–3550.
[13] J. Tomašić, B. Ladešić, Z. Valinger, I. Hršak, Biochim. Biophys. Acta 629 (1980) 77–82.
[14] Z. Valinger, B. Ladešić, J. Tomašić, Biochim. Biophys. Acta 701 (1982) 63–71.
[15] Z. Valinger, B. Ladešić, I. Hršak, J. Tomašić, Int. J. Immunopharmacol. 9 (1987) 325–332.
[16] B. Halassy, M. Krstanović, R. Frkanec, J. Tomašić, Vaccine 21 (2003) 971–976.
[17] D.D. Lasić, Liposomes: From Physics to Applications, Elsevier, Amsterdam, 1993, p. 268.
[18] G.F. Kersten, D.J. Cromemelin, Biochim. Biophys. Acta 1241 (1995) 117–138.
[19] T. Lian, R.J.Y. Ho, J. Pharm. Sci. 90 (2001) 667–679.
[20] J.J. Volwerk, O.H. Griffith, Magn. Reson. Rev. 13 (1988) 135–178.
[21] D. Marsh, T. Pali, L.I. Horvath, in: L. Berliner (Ed.), Spin Labeling: Next Millenium, Biol. Magn. Reson., vol. 14, Plenum, New York, 1998, pp. 23–82.
[22] J.B. Feix, C.C. Klug, in: L. Berliner (Ed.), Spin Labeling: Next Millenium, Biol. Magn. Reson., vol. 14, Plenum, New York, 1998, pp. 251–281.
[23] J.W. Metzger, W.H. Sawyer, B. Wille, L. Biesert, W.G. Bessler, G. Jung, Biochim. Biophys. Acta 1149 (1993) 29–39.
[24] L. Coldeck, J. Fonollosa, M. de Pera, J. Estelrich, A. Dela Maza, J. Control. Release 68 (2000) 85–95.
[25] F. Momo, A. Wisniewska, R. Stevanato, Biochim. Biophys. Acta 1240 (1995) 89–94.
[26] Y. Morimoto, Y. Takeuchi, Chem. Pharm. Bull. 42 (1994) 1890–1895.
[27] E.G. Rozantsev, Free Nitroxyl Radicals, Plenum, New York, 1970, p. 204.
[28] M. Krstanović, R. Frkanec, B. Vranešić, Đ. Ljevaković, V. Šporec, J. Tomašić, J. Chromatogr., B 773 (2002) 167–174.
[29] P.D. Morse, Biophys. J. 51 (1987) 440a.
[30] W.L. Hubbell, H.M. Mc Connell, J. Am. Chem. Soc. 93 (1971) 314–326.
[31] S.P. Van, B. Birrell, H. Griffith, J. Magn. Reson. 15 (1974) 444–459.
[32] O.H. Griffith, P.C. Jost, Spin Labeling in Theory and Practice, Academic Press, New York, 1976, pp. 251–272.
[33] Kivelson, J. Chem. Phys. 33 (1960) 1094.
[34] D. Marsh, Electron Spin Resonance: Spin Labels in Membrane Spectroscopy, Springer Verlag, Berlin, 1981, pp. 52–120.
[35] B. Klaić, Carbohydr. Res. 138 (1985) 65–72.
[36] T. Daemen, B.H.J. Dontje, J. Regts, G.L. Scherphof, J. Immunother. 13 (1) (1993) 31–35.
[37] T. Asano, K. Matsushima, E.S. Kleinerman, Cancer Immunol. Immunother. 38 (1994) 16–22.
[38] J. Turanek, D. Zaluska, A. Vacek, P. Borkovcova, J. Thurnvaldova, L. Blaha, K. Mašek, Int. Immunopharmacol. 1 (2001) 167–175.
[39] G. Baratt, C. Morin, F. Schuber, Liposomal immunomodulators, in: F. Puisieux, P. Couvreur, J. Delattre, J.P. Devissaguet (Eds.), Liposomes, New Systems and New Trends in Their Applications, Editions de Sante, Paris, 1995, pp. 461–506.
[40] M.A.V. Gianan, E.S. Kleinerman, Cancer Biotherapy and Radiopharmaceuticals 13 (5) (1998) 363–368.
[41] J.J. Killion, I.J. Fidler, Pharmacol. Ther. 78 (3) (1998) 141–154.
[42] J. Tomašić, I. Hršak, Biochim. Biophys. Acta 716 (1982) 217–223.
[43] M.B. Sankaram, D. Marsh, in: L. Berliner (Ed.), Spin Labeling: Next Millenium, Biol. Magn. Reson., vol. 14, Plenum, New York, 1998, pp. 5–22.