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European Journal of Pharmaceutics and Biopharmaceutics 57 (2004) 329–336
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Phloretin and 6-ketocholestanol: membrane interactions studied by a phospholipid/polydiacetylene colorimetric assay and differential scanning calorimetry

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Received 23 June 2003; accepted in revised form 26 September 2003

Abstract

The aim of this study was to investigate membrane interactions of phloretin and 6-ketocholestanol using different methods. A previously reported colorimetric assay with phospholipid/polydiacetylene (PDA) vesicles was used to examine a possible interaction of phloretin and 6-ketocholestanol with this target. During this interaction the used aggregates of lipids and conjugated PDA undergo a visible and quantifiable blue to red color transition. A positive result is indicative for a reaction response with membrane lipids of a simplified bilayer structure instead of the complex bilayer system of the stratum corneum. Results of this test confirm previous proposed membrane interactions by skin diffusion studies. Additional differential scanning calorimetry studies with 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) liposomes confirm a membrane interaction and indicates that phloretin and 6-ketocholestanol interact with the lipid layer and change structural parameters. They strongly decrease the lipid phase transition temperature of DMPC and DPPC liposomes by at least about 6.6 °C and maximally about 13.9 °C which refers to a higher fluidity of the membrane.

Keywords: Phloretin; 6-Ketocholestanol; Phospholipids; Polydiacetylene; Membrane partition; Differential scanning calorimetry

1. Introduction

Phloretin and 6-ketocholestanol are two very interesting substances and have been shown to enhance skin permeation of various drugs [1–5]. One of the molecular mechanisms which is currently discussed is the influence of these two substances on the membrane dipole potential [6–9]. Indirect proof could be derived from differential scanning calorimetry (DSC) experiments with native porcine skin in which a shift of the characteristic lipid transition temperature to lower values was observed [2]. This is indicative of a higher fluidity of the membrane. Diffusion experiments are expensive and time-consuming, furthermore the most reliable model, human skin needs special preparation techniques, and is also difficult to obtain in sufficient amounts. Therefore, it would be of great benefit to have additional test systems. Recently, it has been shown that the application of a bio-mimetic lipid/polydiacetylene (PDA) supramolecular assembly for studying such membrane interactions can be an elegant and suitable tool [10]. The lipid/PDA vesicles used represent a simplified membrane model instead of the complex organised lipids of the stratum corneum [11]. It has been demonstrated that currently used penetration enhancers like azone, transcutol or oleic acid induced colour changes after addition to polymerised blue lipid/PDA vesicles. The new test system could provide insight into the mechanisms of permeability of membrane active compounds.

The aim of this study was to investigate the interactions of phloretin and 6-ketocholestanol with 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC)/PDA and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC)/PDA vesicles by quantifying the possible colour changes. Additional DSC experiments with pure lipids should confirm these interactions.

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doi:10.1016/j.ejpb.2003.09.012

2. Materials and methods

2.1. Materials

DPPC and DMPC were purchased from Avanti Polar Lipids (Alabaster, AL). The diacetylene monomer 10,12-tricosadiynoic acid was purchased from GFS Chemicals (Powell, USA). Phloretin (PH), phlorizin, 6-ketocholestanol (KC), trifluorethanol and L-α-phosphatidyl-choline (PC) were obtained from Sigma (St Louis, MO). The chemical structures of PH, phlorizin and KC are presented in Fig. 1. All other chemicals used in the study were of analytical reagent grade and were used as received without any further purification.

2.2. Stock solutions and liposomes

2.2.1. Stock solutions

Stock solutions of 6-ketocholestanol (15 mM) in trifluorethanol, phloretin and phlorizin in methanol (15 mM) were prepared and diluted to the different concentrations (2.5–15 mM) or directly used.

2.2.2. Multilamellar liposomes with PC and phloretin or 6-ketocholestanol

Multilamellar PC-liposomes were prepared with the film method as previously reported but without final extrusion [1]. The concentrations of 6-ketocholestanol and phloretin ranged from 0.5 to 7 mM. The lipid films were re-hydrated with 10 mM phosphate buffer (pH 7.2). Lipid concentration was 1 mg/ml liposomes.

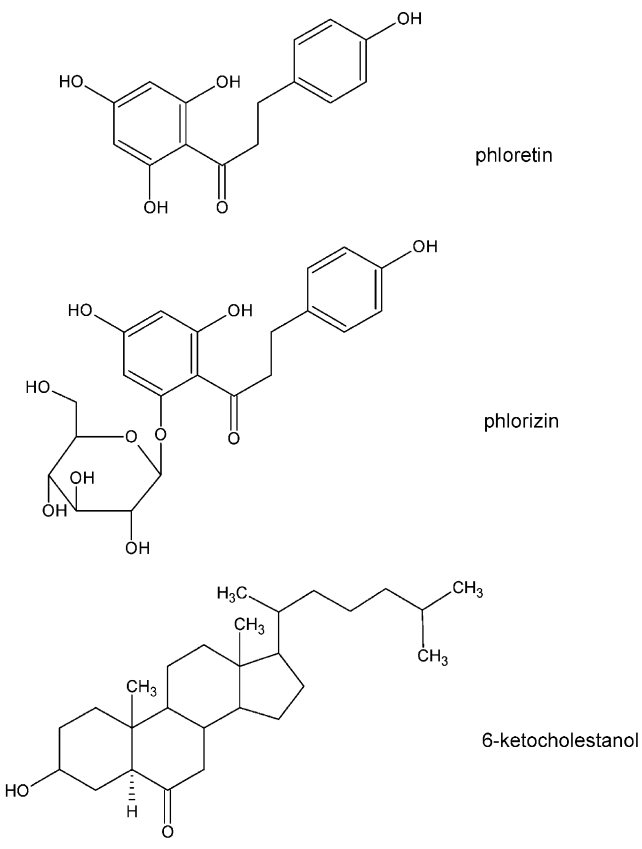

Fig. 1. Chemical structures of phloretin, phlorizin and 6-ketocholestanol.

2.3. Preparation of lipid/PDA vesicles and colorimetric measurement

The vesicles were prepared by probe sonication of an aqueous mixture of synthetic DPPC or DMPC and tricosadiynoic acid at a temperature of approximately 70 °C for 3 min. The molar ratio between the lipid and tricosadiynoic acid was 2:3. Following sonication the solution was cooled to 4 °C overnight and then irradiated at 254 nm for 10–20 s to induce polymerisation of the PDA backbone. The resulting vesicles exhibit an intense blue appearance. They can be stored at 4 °C in the refrigerator until use. The resulting products are designated as phospholipid/PDA vesicles (DPPC/PDA and DMPC/PDA).

2.3.1. Experimental set-up with methanolic and trifluorethanolic solutions

For all tests 50 μl of the phospholipid/PDA vesicles were applied to each well of a 96-well microtiter plate. Afterwards 100 μl of trifluorethanolic solution of 6-ketocholestanol or methanolic solutions of phlorizin or phloretin in different concentrations were added. The pure solvents served as controls.

After incubation of the plate at 27 °C for 10 min the colorimetric response of the blue to red transition within the solutions was estimated at 500 nm for blue and 640 nm for red. A quantitative value for the extent of the blue to red colour transition within the solutions was calculated according to a previous study [12]. In order to quantify the response of a liposome solution to a given amount of penetration enhancer, the visible absorption spectrum of the liposome solution without the penetration enhancer was analysed as

$$PB = A_{blue}/(A_{blue} + A_{red})$$

where A is defined as either the blue component in the UV–Vis spectrum (~640 nm) or the red component (~500 nm). Blue and red refer to the visual appearance of the material and not its actual absorbance. PB_0 is the red/blue ratio of the control sample before induction of a colour change while PB_1 is the value obtained after the colorimetric transition occurs.

The colorimetric response (%CR) is defined as the percentage change in PB upon exposure of the penetration enhancer:

$$\%CR = [(PB_0 - PB_1)/PB_0]100.$$

2.3.2. Experimental set-up with PC-liposomes

For all tests 50 μl of the phospholipid/PDA vesicles were applied to a 96-well microtiter plate. Afterwards 100 μl of

6-ketocholestanol-PC-liposomes and phloretin-PC-liposomes, respectively, were added. The pure PC-liposomes served as controls. The procedure performed with PC-liposomes was identical with the protocol described in Section 2.3.1.

2.4. Samples for DSC-study

DMPC and DPPC liposomes were prepared in the presence and absence of phloretin and 6-ketocholestanol, respectively.

For this purpose 20 mg lipid and 8.5 mg phloretin were dissolved in 1 ml Tris buffer (0.1 M; pH 7.0) and ultrasonicated for 6 min or 20 mg lipid and 6.5 mg 6-ketocholestanol were dissolved in 1 ml Tris buffer (0.1 M; pH 7.0) and ultrasonicated for 6 min. The resulting liposomes were designated as DPPC-phloretin, DMPC-phloretin, DMPC-6-ketocholestanol and DPPC-6-ketocholestanol. DPPC liposomes and DMPC liposomes served as controls.

2.5. DSC-experiments

Aliquots of 20 μl of the vesicles were transferred to a 30 μl DSC aluminium, hermetically sealed pan (Perkin-Elmer, US) and submitted to DSC analysis. The temperature of the maximum of the transition endotherm (T_m) as well as the onset and end of the curve, the area under the curve as measure for the enthalpy and the width at half height of the curve ($T_{1/2}$) as indicator of the peak width were determined by using a Perkin-Elmer DSC-7. The scan rate employed was 0.5 °C/min in the temperature range of 0–70 °C. Tris buffer (pH 7.0) was used in the reference pan.

2.6. Statistical data analysis

The number of experiments are indicated in the figure and table legends.

Results are expressed as the mean ± SD of the experiments. Statistical data analysis was performed using the *t*-test with $P < 0.05$ as a minimal level of significance.

3. Results

A schematic picture of a part of the vesicle assembly used in this study, which consists of PDA and DPPC or DMPC, respectively, is presented in Fig. 2. The vesicles contained approximately 2% of lipids and appeared intense blue to the eye due to the alternating triple bond to double bond conjugated PDA backbone. For example, as demonstrated in a photograph (Fig. 3) prior to addition of 6-ketocholestanol the lipid/PDA vesicles appear blue and after addition appear red. A comparison of the UV–Visspectrum of the pure DPPC/PDA vesicles (Fig. 4A) to that with 6-ketocholestanol (Fig. 4B) the induced blue to red colour shift is visualised. The tests were performed with two lipids DPPC and DMPC, respectively. In Fig. 5 the colorimetric response (CR) caused by addition of increasing amounts of phloretin, phlorizin and 6-ketocholestanol in

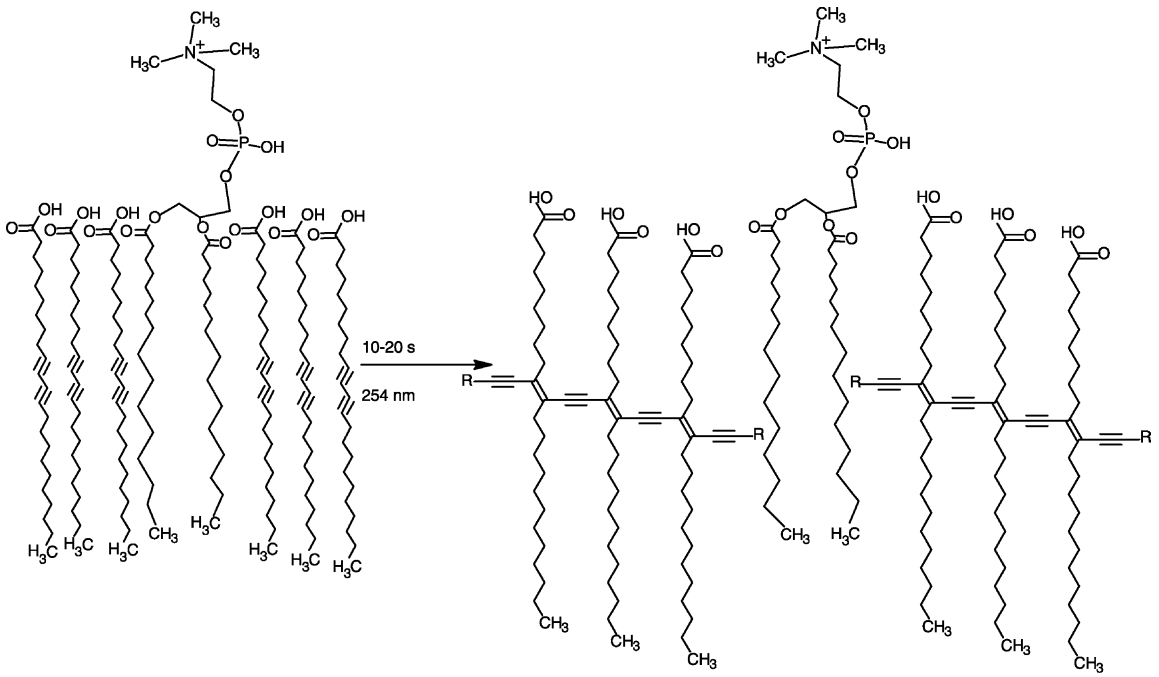

Fig. 2. Schematic picture of a portion of the DMPC/PDA or DPPC/PDA vesicles used in the experiments. The figure shows parts of the vesicle interface which consists of a polymerised network of 10,12-tricosadiynoic acid and inserted phospholipids.

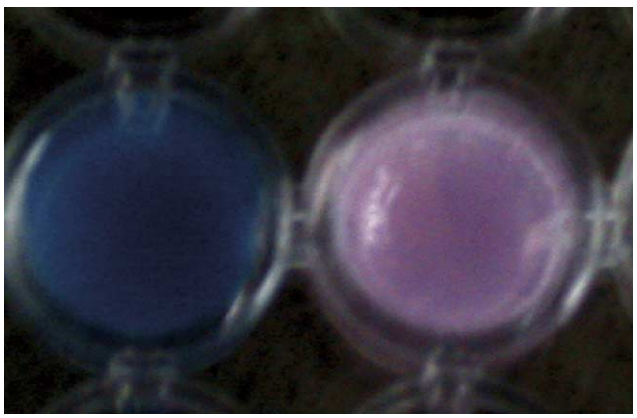

Fig. 3. Photograph of the colour change in DPPC/PDA vesicles on a microtiter plate. Left hand side, prior to 6-ketocholstanol addition; right hand side, after addition of 6-ketocholestanol (15 mM).

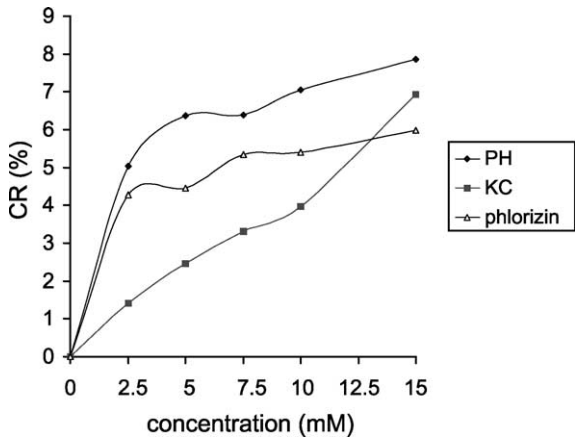

Fig. 5. Colorimetric response (defined in Section 2) of DPPC/PDA vesicles titrated with 6-ketocholestanol in trifluorethanol and phloretin and phlorizin in methanol. The values of pure trifluorethanol and pure methanol are subtracted. Indicated values are means (± SD) of at least three experiments.

DPPC/PDA is shown. In Fig. 6, the CR of the substances in DMPC/PDA is presented. Although the structural difference between the two lipids is very small there are significant differences in the colorimetric response. As can be seen from Fig. 5 there is not a big difference in DPPC/PDA of the CR between phloretin and phlorizin. In contrast, for DMPC/PDA (Fig. 6) a colour change can be observed with phloretin but not with phlorizin. Phlorizin (phloretin-glucoside) is the naturally occurring phloretin 2′-β-*o*-glucoside. This increase in hydrophilicity seems to decrease its interaction with the vesicles.

The CR between the penetration enhancers and the vesicles is higher in DMPC/PDA than in DPPC/PDA vesicles for 6-ketocholestanol, however, only a qualitative statement as to whether an interaction with the phospholipid/PDA vesicles occurs is possible. Interestingly the solvents alone like methanol or trifluorethanol also cause a colour change which is indicative of a type of re-organisation. In all presented figures the CR-values of the solvents are subtracted from the CR-values of penetration enhancer in solvent. In order to minimize the influence of the solvent, the penetration enhancers were incorporated in phospholipid liposomes. For this purpose, loaded liposomes

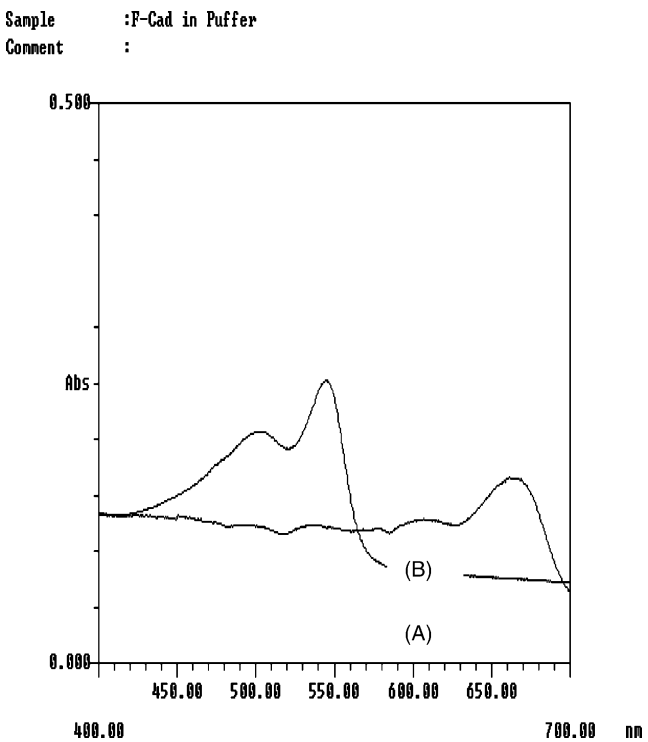

Fig. 4. Colorimetric detection of phloretin by polymerised DPPC/PDA liposomes. (A) Visible absorption spectrum of blue/red liposome solution prior to addition of 6-ketocholestanol. (B) Visible absorption spectrum of DPPC/PDA liposomes after the addition of 6-ketocholestanol to a final concentration of 15 mM.

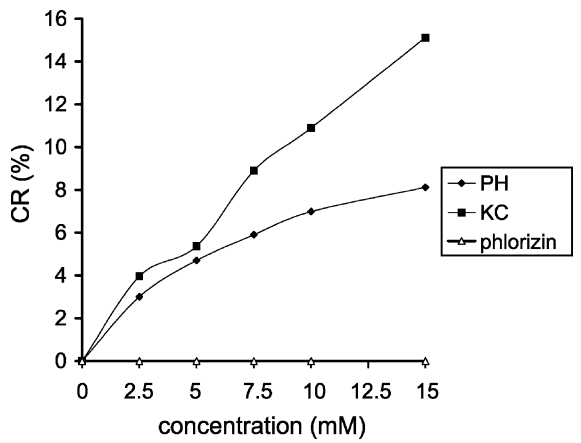

Fig. 6. Colorimetric response (defined in Section 2) of DMPC/PDA vesicles titrated with 6-ketocholestanol in trifluorethanol and phloretin and phlorizin in methanol. The values of pure trifluorethanol and pure methanol are subtracted. Indicated values are means (± SD) of at least three experiments.

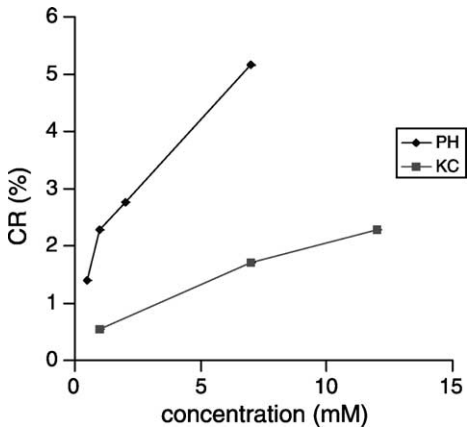

Fig. 7. Colorimetric response (defined in Section 2) of DPPC/PDA vesicles titrated with phloretin and 6-ketocholestanol incorporated in phospholipid liposomes. The values of pure liposomes are subtracted. Indicated values are means (± SD) of at least three experiments.

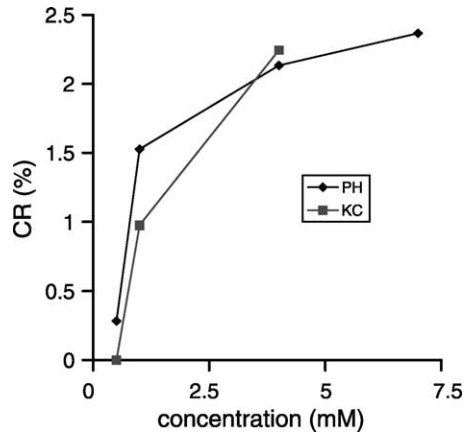

Fig. 8. Colorimetric response (defined in Section 2) of DMPC/PDA vesicles titrated with phloretin and 6-ketocholestanol incorporated in phospholipid liposomes. The values of pure liposomes are subtracted. Indicated values are means (± SD) of at least three experiments.

were added to the blue coloured DPPC/PDA or DMPC/PDA vesicles, respectively. The procedure was the same as with the solutions. The results are presented in Figs. 7 and 8. As can be seen the CR values are generally smaller in both systems but also limited to a concentration in case of 6-ketocholestanol of 4 mM and of phloretin of 7 mM. At higher concentrations the colour test failed. A possible explanation could be the additional interactions of PC and the phospholipd/PDA vesicles. The test should be performed without additional interfering surface active compounds.

The interaction of phloretin and 6-ketocholestanol was also studied by DSC in DMPC and DPPC liposomes. In a previous study [2] the interaction of porcine skin and the two substances was reported. This effect was manifested by a decrease of the main lipid transition temperature T_m of the lipid bilayer. As can be seen from Table 1 the T_m of the DPPC liposomes was 48.2 °C whereas the T_m of DMPC was 31.83 °C. These characteristic transition temperatures were decreased by phloretin as well as by 6-ketocholestanol in both model lipids. In Fig. 9 DSC curves of DMPC compared with DMPC in the presence of phloretin and the DSC curves of DPPC compared with DPPC in the presence of phloretin are shown. Interestingly 6-ketocholestanol also exhibited a similar effect as phloretin. In Fig. 10 DSC scans in the presence of 15 mol% 6-ketocholestanol compared to DMPC and DPPC are shown. As seen the effect of 6-ketocholestanol was more pronounced in DPPC liposomes with a decrease of 13.9 °C. The results of the DSC measurements are summarised in Table 1. The addition of 6-ketochoestanol and phloretin not only changed the phase transition temperature but also led to an increase of the width at half height $T_{1/2}$ which is related to the purity of the system and the co-operativity of the phase transition.

4. Discussion

The physical and chemical basis of the chromatic transitions in PDA-based vesicles are due to interfacial perturbations which induce strains and distortions within the pendant side chains of the PDA. The structural perturbations are believed to give rise to a *gauche–trans* conformational transition of the PDA backbone resulting in shortening of the conjugation network and absorption at a shorter wavelength and accordingly the red appearance compared

Table 1
Influence of phloretin (PH) and 6-ketocholestanol (KC) on the phase transition temperature T_m, enthalpy and $T_{1/2}$ of DMPC and DPPC liposomes, respectively, $n = 5–8$

Lipid	Onset	T_m (°C)	End	$T_{1/2}$ (°C)	Enthalpy
DMPC	31.83 ± 0.13	31.93 ± 0.13	32.02 ± 0.15	0.10 ± 0.01	10.73 ± 0.71
DMPC + PH	21.62 ± 0.08	21.78 ± 0.11	22.10 ± 0.27	0.24 ± 0.07	10.86 ± 1.56
DMPC + KC	21.69 ± 0.19	21.78 ± 0.11	22.10 ± 0.27	0.21 ± 0.07	10.45 ± 1.50
DPPC	48.20 ± 0.15	48.36 ± 0.14	48.56 ± 0.14	0.18 ± 0.01	18.03 ± 1.24
DPPC + PH	41.56 ± 0.26	42.27 ± 0.43	42.58 ± 0.47	0.71 ± 0.20	18.83 ± 3.14
DPPC + KC	34.28 ± 0.01	34.40 ± 0.01	34.62 ± 0.09	0.17 ± 0.01	15.13 ± 1.33

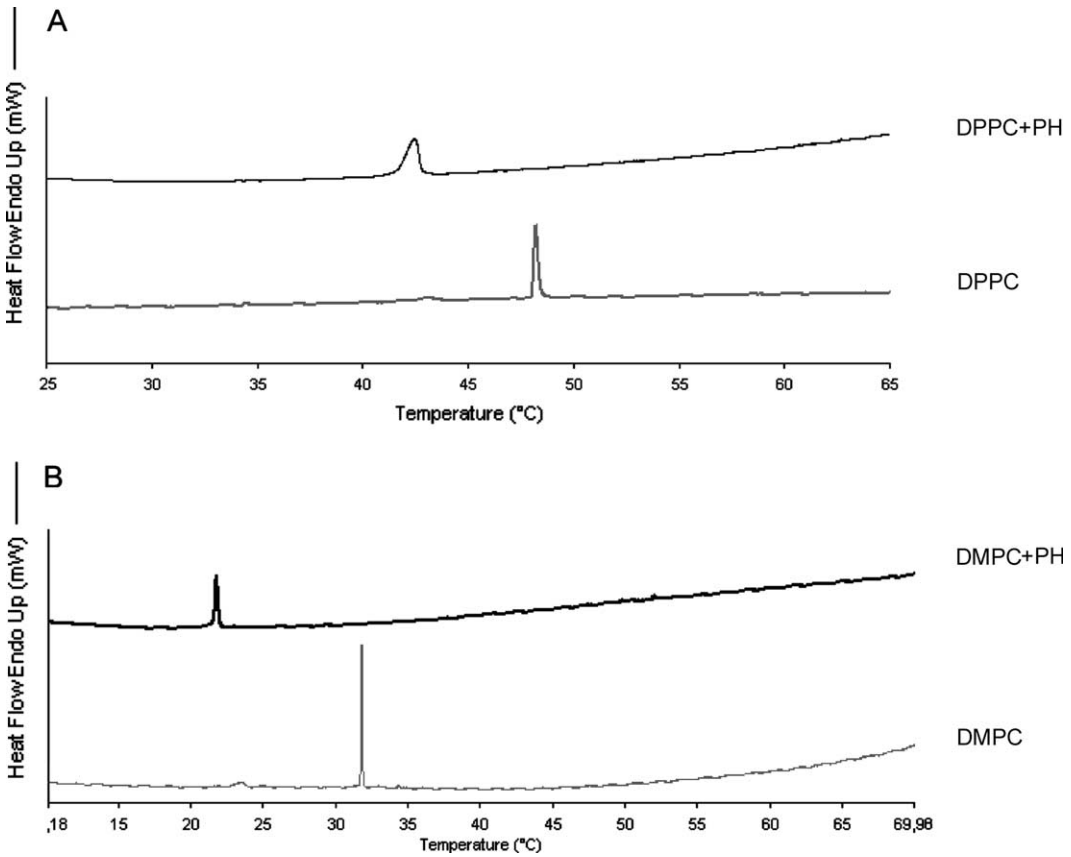

Fig. 9. DSC endotherms (excess heat capacity versus temperature) of lipid multilamellar lipid vesicles. (A) DPPC in comparison with DPPC + PH; (B) DMPC in comparison with DMPC + PH.

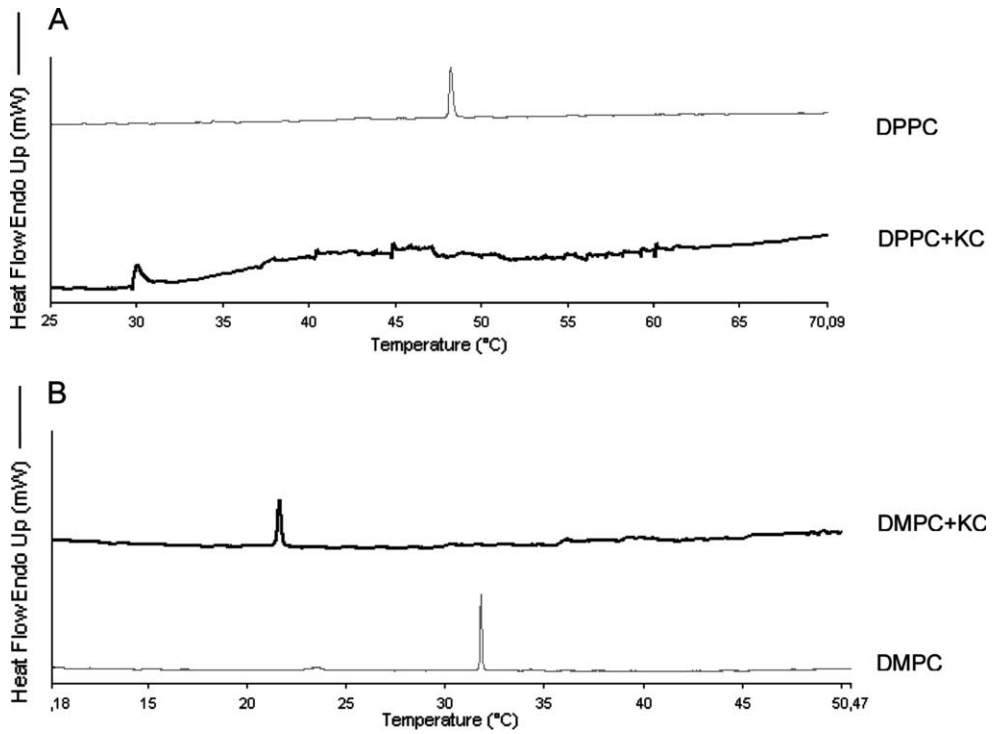

Fig. 10. DSC endotherms (excess heat capacity versus temperature) of lipid multilamellar lipid vesicles. (A) DPPC in comparison with DPPC + KC; (B) DMPC in comparison with DMPC + KC.

to the initial blue colour. It has been reported that the irreversible nature of the blue to red colour transformation is due to a transition from a meta-stable blue phase into a more thermodynamically stable red-phase [13,14]. In order to investigate the chromatic transition of PDA liposomes, the role of headgroup amino acids in promoting colour transition has been studied by probing the colour change of head group derivatised liposomes [15,16]. In these papers the characteristic blue to red chromatic transition of PDA assemblies allows the direct detection of biological targets in a fast test. The interaction-based chromatic transition requires incorporation of molecular recognition units into the PDA assemblies either in a covalent [17,18] or a non-covalent manner [12,19]. In our case concerning the interaction of phloretin and 6-ketocholestanol the binding will be non-covalent. Colorimetric sensors using PDA assemblies for cholera toxin [12,19], influenza virus [17] or *Escherichia coli* [20] have been reported.

The results of the DSC studies correlate with published data which are only available for phloretin and multilamellar DMPC vesicles. In these DMPC vesicles phloretin in its neutral form strongly decreased the lipid phase transition temperature and slightly reduced the co-operativity of the phase transition within the lipid bilayer [21]. The shift in these multilamellar vesicles was similarly high as in our studies and depended on the pH. The transition temperature decreasing effect was dependent on the concentration in the lipid phase and integration into the membrane and changed the lipid packing. We saw a similar effect in DPPC liposomes. For 6-ketocholestanol in both systems a decrease of the lipid transition temperature could be shown. Phloretin and 6-ketocholestanol were shown to modify the binding and translocation rates of hydrophobic ions in lipid vesicle systems in a manner that is consistent with lowering and raising of the internal dipole potential [6]. Results of the colorimetric assay as well as of the DSC-studies indicate a membrane interaction of phloretin and 6-ketocholestanol without considering their reverse dipole potential. Further studies will be necessary. A recent article reports that 6-ketocholestanol forms pores inside a phospholipid monolayer similar to that with channel-forming peptides and proteins [22].

5. Conclusion

Membrane interactions of phloretin, phlorizin and 6-ketocholestanol have been demonstrated by a simple colorimetric test with phospholipid/PDA vesicles. Additional DSC studies on phospholipids vesicles with phloretin and 6-ketocholestanol showing decreased lipid phase transition temperatures confirm these interactions.

Acknowledgements

This work was supported by Grant No. P15137 from the Fonds zur Förderung Wissenschaftlicher Forschung (FWF) to Prof. Dr Claudia Valenta.

References

[1] C. Valenta, J. Cladera, P. O'Shea, J. Hadgraft, Effect of phloretin on the percutaneous absorption of lignocaine across human skin, J. Pharm. Sci. 90 (2001) 485–492.
[2] C. Valenta, M. Nowak, Influence of phloretin and 6-ketocholestanol on the permeation of progesterone through porcine skin, Int. J. Pharm. 217 (2001) 79–86.
[3] J. Cladera, P. O'Shea, J. Hadgraft, C. Valenta, Influence of molecular dipoles on human skin permeability: use of 6-ketocholestanol to enhance the transdermal delivery of bacitracin, J. Pharm. Sci. 92 (2003) 1018–1027.
[4] B.G. Auner, C. Valenta, J. Hadgraft, Influence of lipophilic counter-ions in combination with phloretin and 6-ketocholestanol on the skin permeation of 5-aminolevulinic acid, Int. J. Pharm. 255 (2003) 109–116.
[5] B.G. Auner, C. Valenta, J. Hadgraft, Influence of phloretin and 6-ketocholestanol on the skin permeation of sodium-fluorescein, J. Control. Rel. 89 (2003) 321–328.
[6] J.C. Franklin, D.S. Cafiso, Internal electrostatic potentials in bilayers: measuring and controlling dipole potentials in lipid vesicles, Biophys. J. 65 (1993) 289–299.
[7] B. Bechinger, J. Seelig, Interaction of electric dipoles with phospholipid head groups. A 2H and 31P NMR study of phloretin and phloretin analogues in phosphatidylcholine membranes, Biochemistry 30 (1991) 3923–3929.
[8] J. Cladera, P. O'Shea, Intramembrane molecular dipoles affect the membrane insertion and folding of a model amphiphilic peptide, Biophys. J. 74 (1998) 2434–2442.
[9] G.L. Jendrasiak, R.L. Smith, T.J. McIntosh, The effect of phloretin on the hydration of egg phosphatidylcholine multilayers, Biochim. Biophys. Acta 1329 (1997) 159–168.
[10] D. Evrard, E. Touitou, S. Kolusheva, Y. Fishov, R. Jelinek, A new colorimetric assay for studying and rapid screening of membrane penetration enhancers, Pharm. Res. (2001) 943–949.
[11] P.W. Wertz, M.C. Miethke, S.A. Long, J.S. Strauss, D.T. Downing, The composition of the ceramides from human stratum corneum and from comedones, J. Invest. Dermatol. 84 (1985) 410–412.
[12] J.J. Pan, D. Charych, Molecular recognition and colorimetric detection of cholera toxin by poly(diacetylene) liposomes incorporating Gm1 ganglioside, Langmuir 13 (1997) 1365–1367.
[13] H. Ringsdorf, B. Schlarb, J. Venzmer, Molekulare architektur und funktion von polymeren orientierten systemen—modelle für das studium von organisation, oberflächenerkennung und dynamik von biomembranen, Angew. Chem. 100 (1988) 117–162.
[14] S. Kolusheva, T. Shahal, R. Jelinek, Cation-selective color sensors composed of ionophore–phospholipid–plydiacetylene mixed vesicles, J. Am. Chem. Soc. 122 (2000) 776–780.
[15] Q. Cheng, R.C. Stevens, Charge-induced chromatic transition of amino acid-derivatized polydiacetylene liposomes, Langmuir 14 (1998) 1974–1976.
[16] Q. Cheng, M. Yamamoto, R.C. Stevens, Amino acid terminated polydiacetylene lipid microstructures: morphology and chromatic transition, Langmuir 16 (2000) 5333–5342.
[17] D.H. Charych, J.O. Nagy, W. Spevak, M.D. Bednarski, Direct colorimetric detection of a receptor–ligand interaction by a polymerized bilayer assembly, Science 261 (1993) 585–588.

[18] A. Reichert, J.O. Nagy, W. Spevak, D. Charych, Polydiacetylene liposomes functionalised with sialic acid bind and colorimetrically detect influenza virus, J. Am. Chem. Soc. 117 (1995) 829–830.
[19] D. Charych, Q. Cheng, A. Reichert, G. Kuziemko, M. Stroh, J.O. Nagy, W. Spevak, R.C. Stevens, A litmus test for molecular recognition using artificial membranes, Chem. Biol. 3 (1996) 113–120.
[20] Z. Ma, J. Li, M. Liu, J. Cao, Z. Zou, J. Tu, L. Jiang, Colorimetric detection of *Escherichia coli* by polydiacetylene vesicles functionalized with glycolipid, J. Am. Chem. Soc. 120 (1998) 12678–12679.
[21] R. Cseh, M. Hetzer, K. Wolf, J. Kraus, G. Bringmann, R. Benz, Interaction of phloretin with membranes: on the mode of action of phloretin at the water–lipid interface, Eur. Biophys. J. 29 (2000) 172–183.
[22] L. Becucci, M.R. Moncelli, R. Guidelli, Pore formation by 6-ketocholestanol in phospholipid monolayers and its interpretation by a general nucleation-and-growth model accounting for the sigmoidal shape of voltage–clamp curves of ion channels, J. Am. Chem. Soc. 125 (2003) 3784–3792.