A New Colorimetric Assay for Studying and Rapid Screening of Membrane Penetration Enhancers¥

Damien Evrard,[1] Elka Touitou,[2] Sofiya Kolusheva,[1] Yitzhak Fishov,[3] and Raz Jelinek[1,4]

Received January 24, 2001; accepted April 5, 2001

Purpose. This work aims to demonstrate a novel chemical assay for rapid screening and analysis of the mode of action of membrane interaction by penetration enhancers.

Methods. The new bio-mimetic membrane assembly, consisting of supramolecular aggregates of lipids and conjugated polydiacetylene, undergoes visible and quantifiable blue-red color transitions upon interaction with penetration enhancers.

Results. The new colorimetric model has been employed to examine various classes of penetration enhancers, including 1-dodecylhexahydro-2H-azepin-2-one (Azone), oleic acid, propylene-glycol, menthol, ethoxyglycol-diethyleneglycol-monoethyl-ether (Transcutol), polysorbate-polyethylenesorbitan-monolaurate (Tween-20), and the drug 7-chloro-1-methyl-5-phenyl-3H-1,4-benzodiazepin-2-one (Diazepam). The assay enables to evaluate the validity of various observations and hypotheses proposed in previous studies regarding permeation enhancement activities. Our results suggest, for example, that propylene glycol (PG) by itself does not interfere with membranes, but rather exhibits synergistic effect in combination with other penetration enhancers. Similarly, our data demonstrate that Transcutol does not independently interact with membranes. The colorimetric system also indicates that interaction of penetration enhancers with membranes depend upon the lipid phase, as well as the self-assembly properties of the enhancer molecules.

Conclusions. The new biomimetic model membrane system can be applied for rapid screening of the activities of penetration enhancers, and provides insight into the mechanisms of permeability of membrane-active compounds.

KEY WORDS: penetration enhancers; membrane permeation; biosensors; polydiacetylene.

INTRODUCTION

Lipid assemblies often constitute considerable barriers for drug permeation. This restriction is particularly severe, for example, for percutaneous absorption and drug delivery through the stratum corneum of the skin (1), or insertion of DNA vectors through cellular membranes (2). Various compounds, often referred to as "penetration enhancers," allow faster and easier delivery of drugs through such lipid barriers, and their applications in pharmaceutical formulations and medicinal research have significantly increased in the last two decades (3–5). The molecular mechanisms responsible for the action of penetration enhancers, however, have not been fully elucidated. Proposed models for enhancer activities include interaction with lipid molecules to facilitate diffusion of drug molecules (6), fluidization of bilayer lipids (7), formation of separate phases within the membranes (8), and others (9). Accordingly, development of new model systems for evaluation of the interactions between molecules and lipid assemblies, and for probing mechanisms of membrane permeation would permit screening of potential penetration enhancers, and would significantly contribute to a better understanding of the functions and activities of these compounds.

In this study, we investigate the application of a novel bio-mimetic lipid/polydiacetylene (PDA) supramolecular assembly for studying membrane interactions of penetration enhancers. Previous studies have demonstrated that application of the colorimetric assay yields insight into a variety of membrane-associated events (10–13), accordingly, the experiments described here were designed to examine the applicability of the assay for studying penetration enhancers, and information that could be obtained upon these compounds.

The new colorimetric technique has been applied for studying a series of penetration enhancers individually, in conjunction with each other, and with a drug compound. The experiments were designed to probe the specific effects of the enhancers upon organized lipid assemblies. The compounds examined in this work have been selected to demonstrate the applicability of the new model to a broad range of penetration enhancers, which exhibit distinct activities and different mechanisms. Azone and oleic acid, in particular, have been extensively studied and their permeation enhancement activities are believed to correspond to changes in lipid fluidity (14–16). Transcutol and menthol are similarly presumed to affect the dynamics of lipid molecules within the stratum corneum (17,18). Propylene glycol has been widely used in pharmaceutical formulations, although its exact role as a penetration enhancer has been controversial (19). The permeation activity of the non-ionic surfactant Tween-20 is associated with induction of lipid micellization (20). This work demonstrates that the new colorimetric model can verify previous observations, and provide new information upon membrane interactions of penetration enhancers.

MATERIALS AND METHODS

Materials

Lipids, including dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatydilcholine (DPPC), and ceramide, were purchased from Avanti Polar Lipids (Alabaster, AL). The diacetylene monomer tricosadiynoic acid was purchased from GFS Chemicals (Powell, OH). 1-dodecylhexahydro-2H-azepin-2-one (Azone) was provided to E.T. by Nelson Research (Irvine, CA), trifluoroethanol (TFE), cis-9-octadecenoic acid (oleic acid), trans-9-octadecenoic acid (elaidic acid), propylene-glycol, 5-methyl-2-(1-methylethyl)cyclohexanol (menthol), polysorbate-polyethylenesorbitan-monolaurate (Tween), and 7-chloro-1-methyl-5-phenyl-3H-1,4-benzodiazepin-2-one (diazepam) were purchased from Sigma, ethoxyglycol-diethyleneglycol-

[1] Department of Chemistry and Stadler Minerva Center for Mesoscale Macromolecular Engineering, Ben Gurion University of the Negev, Beersheva 84105, Israel.

[2] Department of Pharmaceutics, Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem 91120, Israel.

[3] Department of Life Science, Ben Gurion University of the Negev, Beersheva, Israel.

[4] To whom correspondence should be addressed. (e-mail: razj@bgumail.bgu.ac.il)

monoethyl-ether (Transcutol) was donated to E.T. by Gattefosse.

Sample Preparation

Lipids were washed in chloroform/water and filtered through 0.8 μm filters. Preparation of vesicular particles containing phospholipids and PDA (2:3 mole ratio) has been carried out as the following: the lipid constituents are dried together *in vacuuo,* followed by addition of de-ionized water and probe-sonication at around 70°C for 2–3 min. The vesicle solution is then cooled at room - temperature, transferred and kept at 4°C overnight, and polymerised using irradiation at 254 nm for 10–20 sec (UV-vis cross-linker). The resulting solution exhibits an intense blue appearance. One mM total lipid (PDA and natural lipid) concentrations were used.

Visible Spectroscopy

Visible spectroscopy measurements were carried out at 27°C on a Hewlett-Packard 8452A diode-array spectrophotometer, using a 1 cm optical path cell. Spectra were acquired at wavelengths between 400 nm and 700 nm. A quantitative value for the extent of the blue-red color transitions within the solutions is given by the colorimetric response (CR), which is defined (11):

$$CR = (PB_0 - PB_I)/PB_0$$

where

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

A is the absorbance at either the "blue" component (~ 640 nm) or the "red" component (~ 500 nm) in the visible spectrum. (Note: 'blue' and 'red' refer to the visual appearance of the material, not its actual absorbance). PB_0 is the red/blue ratio of the control sample (before induction of a color change, however taking into account small color changes induced by pH or temperature), while PB_I is the value obtained after the colorimetric transition occurs.

Fluorescence Measurements

The fluorescence probe 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene-p-toluenesulfonate (TMA-DPH) (Molecular Probes Inc., Eugene, OR) was added to the polymerized vesicles to a final concentration of 1 μM. Addition of the fluorescence probe did not affect both the initial blue color of the vesicles, as well as the color transitions induced by the penetration enhancers. The vesicle solutions containing the fluorescent probe were allowed to equilibrate at 27°C for at least 1 h before the fluorescence measurements.

Steady-state fluorescence anisotropy was measured at a wavelength of 430 nm and λ_{ex} of 360 nm, using a Perkin Elmer LS50B spectrofluorimeter. The control sample did not contain the fluorescent probe. Contribution of light scattering to fluorescence intensity was confirmed to be less than 5%. The temperature was maintained at 27°C. The reported anisotropy values are average of five independent measurements, using three different samples. The fluorescence anisotropy is defined:

$$A = (I_v - I_h)/(I_v + 2I_h)$$

where I_v and I_h are the vertically-polarized and horizontally-polarized fluorescence intensities, respectively.

Transmission Electron Microscopy

Samples were placed on carbon-stabilized copper grids for 1 min, and after removal of excess solution were stained with 1% uranyl acetate. Dried samples were viewed under Philips CM-12 transmission electron microscope at a 100 kV accelerating voltage.

RESULTS

The colorimetric platform we have developed consists of a supramolecular assembly of lipids and polydiacetylene (PDA). PDA aggregates are formed through topotactic polymerization of diynoic acid monomers, and display unique color transitions (21). In particular, PDA aggregates readily undergo blue-red color changes, induced by a variety of external factors, such as temperature (22), changes in pH and salt concentration (23), and interfacial ligand-receptor binding (21). Recent studies have shown that natural membrane lipids embedded within the PDA framework mimic, in effect, lipid-bilayer environments, thus allowing colorimetric detection of lipophilic enzyme catalysis (10), peptide-membrane interactions (11), ion transport (12), and antigen-antibody recognition (13). These studies have demonstrated that biochemical events leading to physical or chemical disruption of the lipid assembly give rise to blue-red colorimetric transitions, induced through structural perturbations of the adjacent PDA network.

Figure 1A depicts the blue-red color change observed upon addition of oleic acid to a cell within a 96-well plate containing dipalmitoylphosphatydilcholine (DPPC)/PDA particles. The color change occurs within a very short time (seconds) after mixing the lipid/polymer particles with the penetration enhancer. The extent of colorimetric transition undergone by the lipid/PDA solution can be evaluated through recording the uv-vis absorbance spectra before and after addition of the compound inducing the color change. Figure 1B, for example, depicts the uv-vis spectra corresponding to the solutions photographed in the wells shown in Fig. 1A. Specifically, comparison of the relative intensities of the absorbance at 500 nm (the "red" band), and the absorbance at 640 nm (the "blue" band), allows a quantitative determination of the degree of the blue-red colorimetric transition, through calculation of a parameter denoted "colorimetric response" (CR) (11). In essence, a higher CR value indicates a stronger reddish appearance of the solution, compared to the blue control sample. For example, the CR calculated for the red solution in the cell shown in Fig. 1B is around 80% (the initial blue solution yields, by definition, a 0%CR).

Figure 1C demonstrates the quantitative application of the new technique, and provides evidence for the chemical and pharmaceutical reliability of the colorimetric model. The titration curves depicted in Fig. 1C compare the relationships between concentrations and colorimetric responses induced by oleic acid (cis-9-octadecenoic acid), and elaidic acid (trans-9-octadecenoic acid). The two monoenoic acids differ only in the relative chain orientation at the double bond, however elaidic acid exhibits significantly smaller penetration

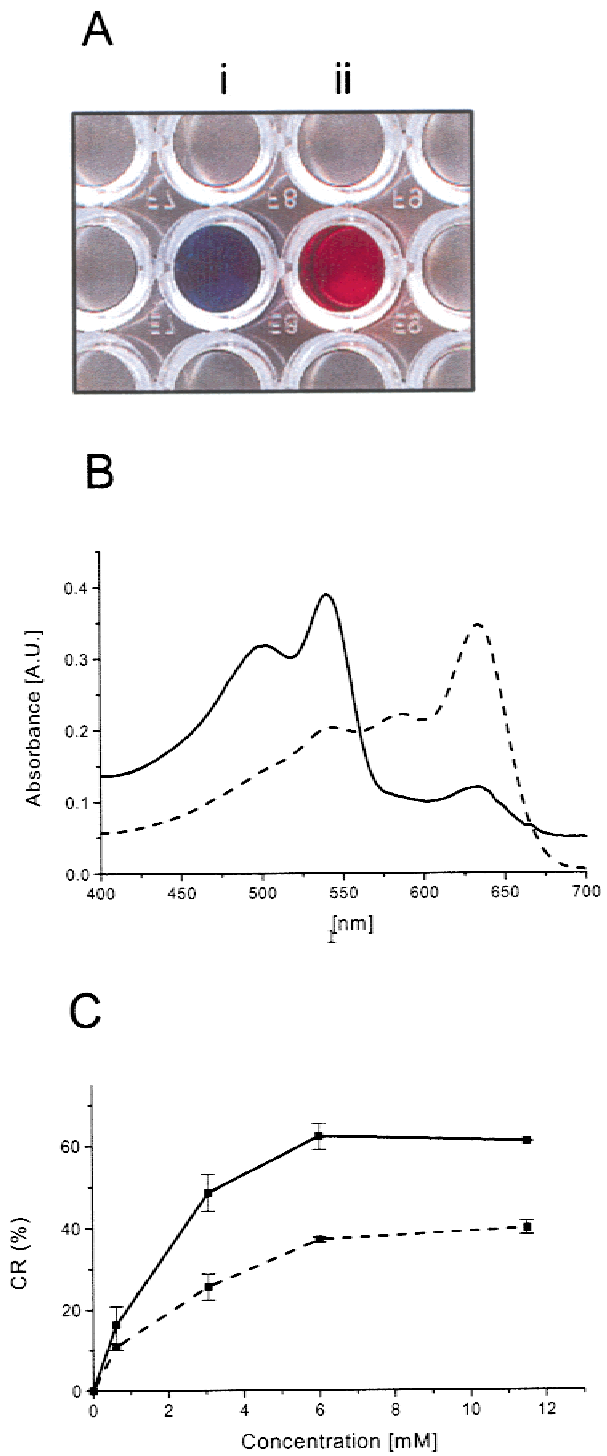

Fig. 1. (A) Cells within the 96-well plate containing: i. 1 mM DPPC/PDA particle solution mixed with tris buffer pH 7.5; ii. 1mM DPPC/PDA solution after addition of oleic acid (20 μM); (B) visible spectra acquired for the solutions shown in (A). Dashed line: control DPPC/PDA solution; solid line: DPPC/PDA solution after addition of oleic acid. (C) Titration curves depicting the percentage colorimetric responses (CR) of DPPC/PDA particle solutions to addition of oleic acid (solid line); elaidic acid (dashed line).

enhancement capabilities, compared to oleic acid (24). The data shown in Fig. 1C are consistent with these pharmaceutical observations. The titration curves depicting the colorimetric responses induced by the two fatty acids, respectively, indicate that the color transitions induced by oleic acid are more significant as compared to elaidic acid. Because the color transitions induced in the lipid/PDA assemblies arise from lipid interference by the added compounds, this result confirms that elaidic acid exhibits less pronounced interactions with the lipid/PDA assemblies.

Figure 2 features titration curves of CR vs. concentration of various penetration enhancers, recorded in solutions containing DMPC/PDA and ceramide/PDA, respectively. Ceramide is the main lipid component of the stratum corneum (3). Both lipid/polymer assemblies exhibit similar relative chromatic responses to the penetration enhancers. The measurements depicted in Fig. 2 are significant. First, the results indicate that the system retains its sensitivity using different lipid models, such as phospholipids (Fig. 2A) or ceramide (Fig. 2B).

The graphs shown in Fig. 2 further reveal that the penetration enhancers examined induce different colorimetric responses. These results indicate that the interactions occurring between the various compounds and the lipid assemblies are not identical. This inference is due to the correlation between the extent of lipid perturbation and the colorimetric transitions observed in lipid/PDA assemblies (12,13). An important observation apparent in Fig. 2 is the almost complete absence of colorimetric response following addition of propylene gly-

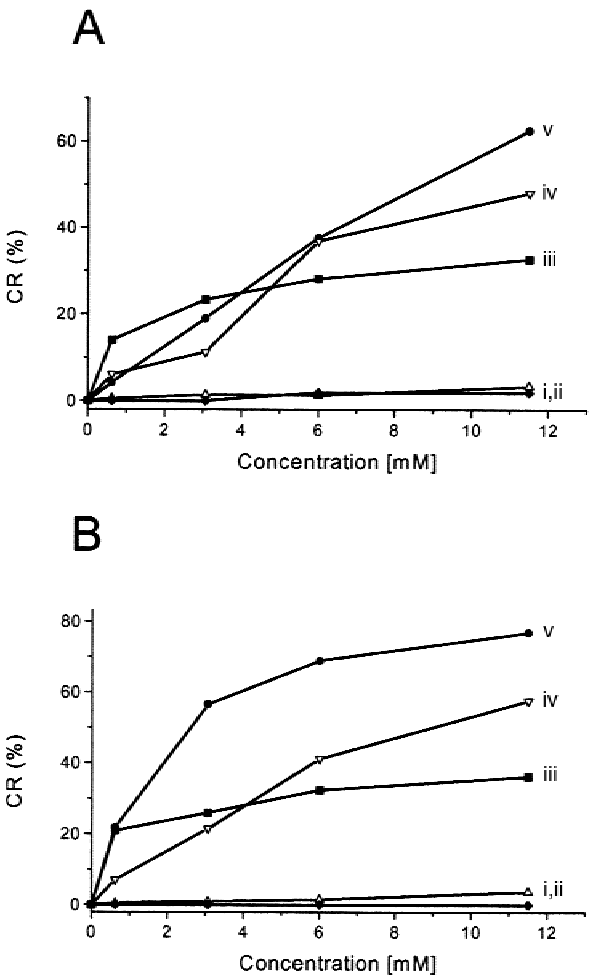

Fig. 2. Titration curves depicting the CR recorded for solutions of: (A) DMPC/PDA; (B) ceramide/PDA, after addition of different compounds. i. Transcutol; ii. PG; iii. Menthol; iv. Azone; v. oleic acid.

col (PG) or Transcutol. The lack of color change, recorded for both lipid models, indicates that both compounds do not significantly interfere with the lipid moieties.

Figures 3–6 depict applications of the new colorimetric assay to analyse a variety of penetration enhancer systems. Fig. 3 presents the colorimetric response, and fluorescence anisotropy data, acquired for DMPC/PDA particles folllowing interaction with Azone, dissolved in trifluoroethanol (TFE), and in PG, respectively. The titration curves displayed in Fig. 3A clearly indicate that the Azone/PG mixture induces significantly higher blue-red colorimetric transitions, compared to Azone dissolved in TFE. Similar data have been obtained after addition of Azone/TFE and Azone/PG to ceramide/PDA (data not shown).

The higher chromatic response of DMPC/PDA upon addition of Azone/PG, compared to Azone/TFE, as depicted in Fig. 3A, is most likely ascribed to the synergy effect attributed to PG (25,26). This synergy is apparent in the greater interference of Azone with the lipid moieties in the presence of PG. To corroborate this hypothesis, and to illuminate the mechanism of Azone interactions with the lipid/polymer particles, we carried out fluorescence anisotropy measurements

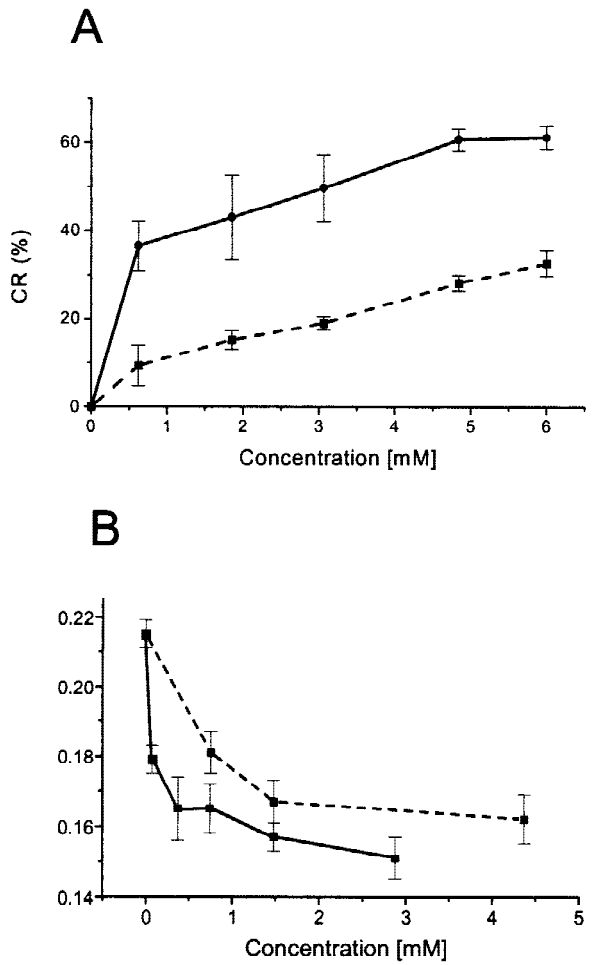

Fig. 3. (A) Titration curves depicting the CR of DMPC/PDA aqueous solutions after addition of Azone in TFE (dashed curve) and Azone in PG (solid curve). (B) Graph showing the dependence of fluorescence anisotropy of TMA-DPH in DMPC/PDA particles on concentrations of Azone/TFE (dashed curve) and Azone/PG (solid curve) in the solution.

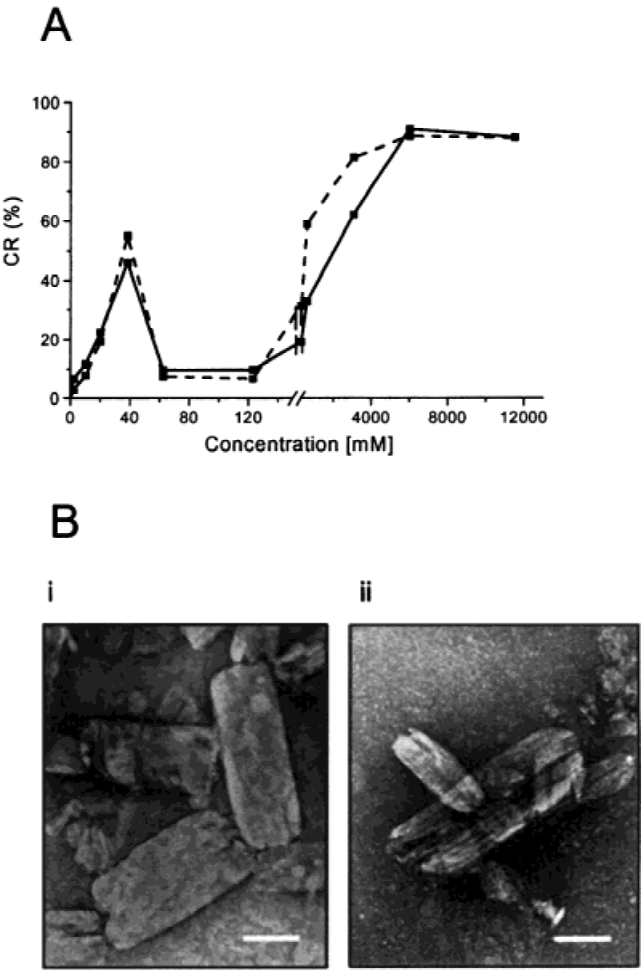

Fig. 4. (A) Titration curves depicting the CR of DMPC/PDA (solid line) and ceramide/PDA (dashed line) after addition of Tween-20. (B) Negative-stained TEM images of: i. DMPC/PDA particles; ii. DMPC/PDA particles after addition of Tween-20 (20 μM).

using the fluorescence probe TMA-DPH, incorporated within the DMPC/PDA particles, Fig. 3B. TMA-DPH has been extensively used to probe the effect of penetration enhancers upon the fluidity of lipid assemblies (27). Fluorescence emission spectra have confirmed that the fluorescence probe is incorporated only within the lipid domains, rather than inside the polymer framework (data not shown). Previous measurements of the changes of fluorescence anisotropy of a similar DPH derivative within DMPC/PDA vesicular particles have confirmed a correlation between the chromatic transitions and the insertion of guest molecules into the lipid moieties (12).

Figure 3B clearly shows that the fluorescence anisotropy of TMA-DPH within the vesicles decreases more rapidly following addition of Azone/PG, compared to Azone/TFE. Because the fluorescence probe TMA-DPH is anchored close to the lipid head-groups (24), the data shown in Fig. 3B point to higher mobility at the lipid/water interface following addition of Azone/PG. The correlation between the increased motion and Azone addition suggests that Azone strongly interacts with the lipid assemblies, in effect inducing higher fluidity closer to the lipid interface. The significant interference of Azone also accounts for the observed colorimetric transitions,

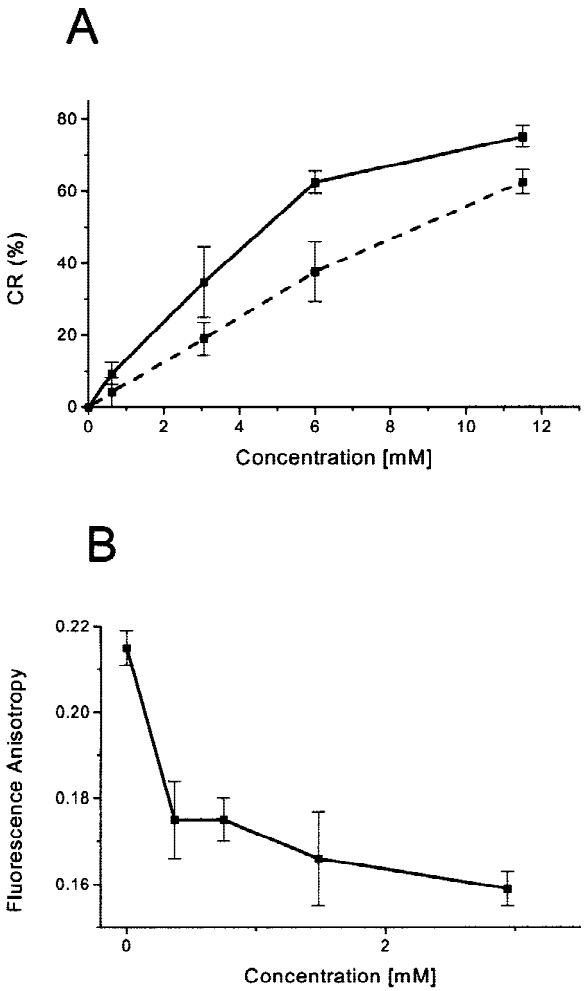

Fig. 5. (A) Titration curves depicting the CR of DMPC/PDA particles (dashed line) and DPPC/PDA particles (solid line) after addition of oleic acid. (B) A graph showing the dependence of fluorescence anisotropy of TMA-DPH in DMPC/PDA particles upon concentration of oleic acid in the solution.

as the PDA framework is highly sensitive to structural perturbation at the lipid surface (13,28). Furthermore, the enhanced fluidity at the lipid interface is most likely an important factor contributing to the penetration enhancing properties of Azone. These data also strengthen previous observations that the co-solvent is important for the enhancing capability of Azone, and that the binary system Azone/PG is more effective than Azone alone (3).

Further confirmation of the apparent relationship between the chromatic transitions and the mode of action of penetration enhancers is provided in Fig. 4. Figure 4A depicts the colorimetric responses of DMPC/PDA and ceramide/PDA assemblies, respectively, following addition of Tween-20, a non-ionic surfactant previously applied as a penetration enhancer (20). In both lipid systems, the titration curves display a local maximum at a concentration of around 40 μM, which is close to the critical micelle concentration (CMC) of Tween-20, which is 59 μM (20). This result is significant, because it indicates that Tween-20 monomers are more membrane-active, compared to the micellar form of the material, in concentrations above the CMC. The data presented in Fig. 4A demonstrate the applicability of the new colorimetric assay for evaluation of actual parameters (such as concentration) affecting penetration enhancement by the compounds examined.

Figure 4B depicts transmission electron microscopy (TEM) images of the lipid/PDA particles that illuminate the morphological and structural effects of Tween-20. Figure 4B(i) features a TEM image of the control sample, containing untreated DMPC/PDA particles. The particles resemble rectangular sheets, which are similar to other modified PDA matrices previously observed in microscopy experiments (29). Addition of Tween-20 (20 μM) clearly affects the surface and overall morphology of the aggregates, as shown in Fig. 4B(ii). In particular, the surfaces of the aggregates become highly fissured, with appearance of oriented grooves corresponding to the conjugated PDA backbone (10,29). The substantial interfacial modification of the lipid/PDA particles is most likely due to specific interactions of Tween-20 with the lipid moieties, resulting in exposure of the oriented PDA framework. Similar interfacial modification induced by biological events at lipid/PDA surfaces has been previously detected (13).

Figure 5 features the concentration-dependent colorimetric responses of oleic acid added to PDA particles incorporating DMPC and DPPC, respectively. This experiment has been carried out to examine the relationship between membrane interactions of the penetration enhancers and the lipid components of the colorimetric assembly. The titration curves depicted in Fig. 5A demonstrate that more pronounced blue-red transitions occur upon addition of oleic acid to DPPC/PDA, compared with DMPC/PDA. This observation is most likely related to the fact that DMPC exhibits a fluid phase at 27°C, whereas DPPC exists in a gel phase at that temperature (30). The data shown in Fig. 4A confirm previous reports indicating that oleic acid exhibits better association and penetration enhancing activities upon interactions with lipids in the gel phase (8,16). Moreover, Fig. 4A demonstrates the significance of the specific interactions between the penetration enhancers and the lipid moieties, rather than the PDA matrix.

Earlier work has suggested that oleic acid induces drug permeation through fluidization of the lipid membrane (8,16). The colorimetric data, combined with fluorescence anisotropy results presented in Fig. 5B, support this hypothesis. Fig-

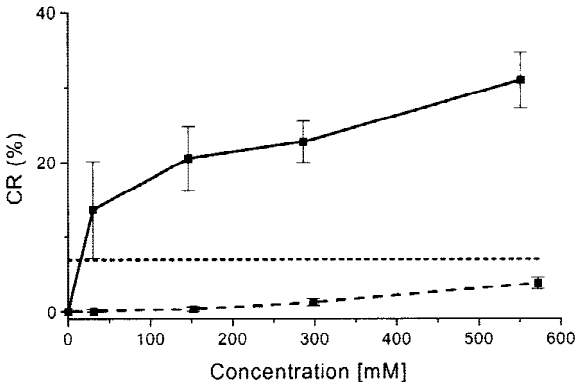

Fig. 6. Titration curves depicting the CR of DMPC/PDA particles after addition of: Transcutol (dashed line); diazepam (dotted line); Transcutol + diazepam (solid line, constant concentration of 100 mM).

ure 5B depicts a graph showing a decrease of the fluorescence anisotropy of TMA-DPH following addition of oleic acid to the DMPC/PDA particles. The anisotropy data indeed indicate that the lipid domains become more fluid (i.e., higher mobility and smaller anisotropy of the fluorescence probe) as the concentration of oleic acid is increased. The higher fluidity of the lipids, which is induced by oleic acid, is most likely the primary factor contributing to the colorimetric transitions. This situation is similar to the case of Azone, see discussion on Fig. 3 above.

Further evidence for the utility of the new colorimetric model is provided in Fig. 6, which depicts the colorimetric responses of the lipid/PDA system to the penetration enhancer Transcutol and the hydrophobic anxiolytic drug diazepam. Transcutol, a monoethyl ether of diethylene glycol, was reported to enhance drug deposition into the stratum corneum through increasing the solubility of the drug in this layer of the skin (31). The results obtain with the colorimetric model furnish critical insight into the activity of Transcutol. Specifically, the titration curves recorded for Transcutol and diazepam separately (Fig. 6, broken lines), indicate negligible interactions between the two individual compounds and the lipid assembly. However, increasing the concentration of Transcutol *in the presence* of diazepam in the solution induces dramatic color changes within the DMPC/PDA particle solution (solid line).

The behaviour depicted in Fig. 6 indicates the existence of a synergistic effect between Transcutol and diazepam. Individually, each molecule does not interact with the membrane. However, our results suggest that diazepam, a lipophilic molecule, interferes strongly with the membrane in the presence of Transcutol. Further experiments will be required to determine the exact mechanism of membrane interaction by Transcutol and diazepam. The response of the assay in this experiment corresponds well to previous reports showing that skin permeation flux of various drugs was enhanced *only* by addition of the binary system Transcutol/oleic acid, but not by Transcutol alone (32). The colorimetric data obtained for Transcutol again point to the potential application of the assay for screening and analysis of penetration enhancers.

DISCUSSION

We present a novel chromatic supramolecular assembly of lipids and polydiacetylene, which facilitates both rapid screening, as well as analysis of the activity of penetration enhancers. The lipid/PDA aggregates successfully mimic organized lipid systems, and exhibit a significant sensitivity toward the interactions between the penetration enhancers and the lipid molecules, through easily detected, rapid color changes. The colorimetric model further permits a quantitative determination of enhancer activities, thus allowing comparison of membrane interactions between different, as well as structurally-related compounds.

The dramatic blue-red transitions induced by the interactions between penetration enhancers and the lipid/PDA assemblies can be ascribed to reorganization of the conjugated network of the polymer backbone (20,27). In binary lipid/PDA systems, in particular, we have previously shown that disruption of the lipid phase by membrane-active molecules can also affect the PDA framework and give rise to colorimetric changes (10,11,13). The colorimetric transformations in such systems are associated with molecular reorganization within the lipid domains, which further perturbs the PDA backbone through the interface separating the components (13). Here, interactions between the penetration enhancers and the lipid moieties give rise to similar structural disruption within the PDA framework, consequently giving rise to the observed colorimetric transitions.

The experiments described in this work indicate that the colorimetric model could be used for detailed analyses of membrane interactions by penetration enhancers. In particular, the significance of the data lies in the apparent correlation between the chromatic responses of the lipid/PDA assemblies, and the activities and mechanisms of membrane interactions of the penetration enhancers. The observation of the stronger colorimetric transitions induced by oleic acid compared to elaidic acid, is consistent with the higher penetration enhancement detected for the latter isomer (23). Similarly, the chromatic technique confirms the relationship between the phase of the lipid assembly (for example fluid phase or gel phase), and the membrane activities of the permeation enhancers. The sensitivity of the colorimetric system to the lipid phase has been demonstrated here for oleic acid.

Use of the chromatic platform has further detected the synergy effects of both propylene glycol (PG), as well as Transcutol. The observation that PG perturbs the lipid assemblies only in combination with other compounds is particularly important. There has been a controversy in the literature regarding the actual role of PG as a penetration enhancer (18,24,25). The colorimetric results obtained here strongly suggest that PG alone does not disrupt the membrane, and does not affect its properties. However, our data indicate that when PG is added in conjunction with other permeation enhancers the situation is different, and a significant perturbation of the lipid assembly is observed. Similar synergy effects were clearly apparent when Transcutol and diazepam have been added to the lipid/PDA solution.

The new colorimetric technique yields useful information upon the mechanisms of membrane disruption by the penetration enhancers. The extent of color transitions has been shown to correlate with an increase in fluidity at the lipid interface, induced by penetration enhancers, such as Azone and oleic acid. The data obtained for Tween-20 are particularly intriguing, indicating that this penetration enhancer surfactant is much less membrane-active in its micellar form, compared to the monomeric structure.

The induced color changes occur rapidly after addition of the penetration enhancers to the solutions containing the lipid/PDA particles. The chromatic assembly is robust and can be easily expanded to include a variety of membrane models; vesicles can incorporate phospholipids with various head-groups, glyco-lipids, sterols, and other membrane constituents. The new biomimetic model membrane system might easily be applied for rapid screening of the activities of penetration enhancers, and could illuminate the mechanisms of permeability of membrane-active compounds.

ACKNOWLEDGMENTS

The authors are grateful to Dr. E. Rahamim (Hebrew University Medical School) for assistance with the TEM experiments, and Prof. J. Hirsch (Tel Aviv University) for the

fluorescence measurements. Prof. Elka Touitou is also affiliated with David R. Bloom Center for Pharmacy, The Hebrew University of Jerusalem, Jerusalem, Israel.

REFERENCES

1. H. Schaefer and T. E. Redelmeier. *Skin Barrier. Principles of Percutaneous Absorption*, Karger, Basel, 1996.
2. E. Wagner. Effects of membrane-active agents in gene delivery. *J. Control. Release* **53**:155–158 (1998).
3. V. R. Goskonda, R. A. Hill, M. A. Khan, and I. K. Reddy. Permeability of chemical delivery systems across rabbit corneal (SIRC) cell line and isolated corneas: A comparative study. *Pharm. Dev. Technol.* **5**:409–416 (2000).
4. A. F. El-Kattan, C. S. Asbill, and B. Michniak. The effect of terpene enhancer lipophilicity on the percutaneous permeation of hydrocortisone formulated in HPMC gel systems. *Int. J. Pharm.* **198**:179–189 (2000).
5. E. Touitou, B. Godin, and C. Weiss. Enhanced Delivery of Drugs Into and Across the Skin by Ethosomal Carriers. *Drug Devel. Res.* **50**:406–415 (2000).
6. Y. Kaplun-Frischoff and E. Touitou. Testosterone skin permeation enhancement by menthol through formation of eutectic with drug and interaction with skin lipids. *J. Pharm. Sci* **86**:1394–1399 (1997).
7. C. L. Gay, T. M. Murphy, J. Hadgraft, I. W. Kellaway, J. C. Evans, and C. C. Rowlands. An electron spin resonance study of skin penetration enhancers. *Int. J. Pharm.* **49**:39–45 (1989).
8. B. Ongpipattanakul, R. R. Burnette, R. O. Potts, and M. L. Francoeur. Evidence that oleic acid exists in a separate phase within stratum corneum lipids. *Pharm. Res.* **8**:350–354 (1991).
9. B. W. Barry. Lipid protein partitioning theory of skin penetration enhancement. *J. Control. Release* **15**:237–248 (1991).
10. S. Y. Okada, R. Jelinek, and D. H. Charych. Induced color change of conjugated polymeric vesicles by interfacial catalysis of phospholipase A-2. *Angew. Chemie, Intl. Ed. Eng.* **38**:655–659 (1999).
11. S. Kolusheva, L. Boyer, and R. Jelinek. Colorimetric assay for rapid screening of anti-microbial peptides. *Nature Biotechnology* **18**:225–227 (2000).
12. S. Kolusheva, T. Shahal, and R. Jelinek. Cation-selective color sensors composed of ionophore–phospholipid–polydiacetylene mixed vesicles. *J. Am. Chem. Soc.* **122**:776–780 (2000).
13. S. Kolusheva, R. Kafri, M. Katz, and R. Jelinek. Rapid colorimetric detection of antibody-epitope recognition at a bio-mimetic membrane interface. *J. Am. Chem. Soc.* **123**:417–422 (2001).
14. C. A. Philips, and B. Michniak. Transdermal delivery of drugs with differing lipophilicities using Azone analogs as dermal penetration enhancers. *J. Pharm. Sci.* **84**:1427–1433 (1995).
15. E. Touitou and B. Fabin. Altered skin permeation of a highly lipophilic molecule. *Int. J. Pharm.* **43**:9–15 (1988).
16. A. Naik, L. A. R. M. Pechtold, R. O. Potts, and R. H. Guy. Mechanism of oleic acid induced skin penetration enhancement in vivo in humans. *J. Control. Release* **37**:299–306 (1995).
17. J. E. Harrison, A. C. Watkinson, D. M. Green, J. Hadgraft, and K. Brain. The relative effect of Azone and Transcutol on permeant diffusivity and solubility in human Stratum Corneum. *Pharm. Res.* **13**:542–546 (1996).
18. J. R. Kunta, V. R. Goskonda, H. O. Brotherton, M. A. Khan, and I. K. Reddy. Effect of menthol and related terpenes on the percutaneous absorption of propanolol across excised hairless mouse skin. *J. Pharm. Sci.* **86**:1369–1373 (1997).
19. J. A. Bouwstra, M. A. de Vries, G. S. Gooris, W. Bras, J. Brussee, and M. Ponec. Thermodynamic and structural aspects of the skin barrier. *J. Control. Release* **15**:209–220 (1991).
20. P. P. Sarpotdar and J. L. Zatz. Percutaneous absorption enhancement by nonionic surfactants. *Drug Develop. Indus. Pharm.* **12**: 1625–1647 (1986).
21. D. H. Charych, J. O. Nagy, W. Spevak, and M. D. Bednarski. Direct colorimetric detection of a receptor-ligand interactions by a polymerized bilayer assembly *Science* **261**:585–588 (1993).
22. H. Tanaka, M. A. Gomez, A. E. Tonelli, and M. Thakur. Thermochromic phase transitions of a polydiacetylene, poly(ETCD), studied by high resolution solid state C-13 NMR. *Macromolecules* **22**:1208–1215 (1989).
23. R. R. Chance. Chromism in polydiacetylene solutions and crystals. *Macromolecules* **13**:386–392 (1980).
24. G. M. Golden, J. E. McKie, and R. O. Potts. Role of stratum corneum lipid fluidity in transdermal drug flux. *J. Pharm. Sci.* **76**:25–28 (1987).
25. N. V. Sheth, D. J. Freeman, W. I. Higuchi, and S. L. Spruance. The influence of Azone, propylene glycol and polyethylene glycol on *in vitro* skin penetration of trifluorothymidine. *Int. J. Pharm.* **28**:201–209 (1986).
26. E. Touitou and L. Abed. Effect of propylene glycol, Azone and n-decylmethyl sulphoxide on skin permeation kinetics of 5-fluoroacil. *Int. J. Pharm.* **27**:89–98 (1985).
27. T. M. Turunen, A. Urtti, P. Paronen, K. L. Audus, and J. H. Rytting. Effect of some penetration enhancers on epithelial membrane lipid domains: evidence from fluorescence spectroscopy studies. *Pharm. Res.* **11**:288–294 (1994).
28. S. Y. Okada, S. Peng, W. Spevak, and D. H. Charych. Color and chromism of polydiacetylene vesicles. *Accounts Chem. Res.* **31**: 229–239 (1988).
29. Q. Cheng, M. Yamamoto, and R. C. Stevens. Amino-acid terminated polydiacteylene lipid microstrcutures: Morphology and chromatic transitions. *Langmuir* **16**:5333–5342 (2000).
30. M. Shinitzky (Ed.) *Biomembranes, Physical Aspects*, John Wiley & Sons, New York, 1993.
31. W. A. Ritschel, R. Panchagnula, K. Stemmer, and M. Ashraf. Development of an intracutaneous depot for drugs. Binding, drug accumulation and retention studies, and mechanism of depot. *Skin Pharmacol.* **4**:235–245 (1991).
32. E. Touitou, F. Levi-Schafer, N. Shaco-Ezra, R. Ben-Yossef, and B. Fabin. Enhanced permeation of theophylline through the skin and its effect on fibroblast proliferation. *Int. J. Pharm.* **70**:159–166 (1991).