Pharmaceutical Research, Vol. 23, No. 3, March 2006 (© 2006)
DOI: 10.1007/s11095-006-9569-1

Research Paper

Rapid Colorimetric Screening of Drug Interaction and Penetration Through Lipid Barriers

Marina Katz,[1] Izek Ben-Shlush,[1] Sofiya Kolusheva,[1] and Raz Jelinek[2,3]

Received June 17, 2005; accepted November 21, 2005

Purpose. The aims of this study are to develop a rapid colorimetric assay for evaluating membrane interactions and penetration through lipid barriers and to create a platform, amenable to high-throughput screening formats, for predicting the extent of penetration of pharmaceutical compounds through lipid layers.
Methods. The colorimetric platform comprises vesicles of phospholipids and the chromatic lipid–mimetic polymer polydiacetylene. The polymer undergoes visible, concentration-dependent blue–red transformations induced through interactions of the vesicles with the molecules examined.
Results. We observe rapid colorimetric transitions induced by addition of pharmaceutical compounds to the chromatic vesicle solutions. We find that the concentration ranges for which the color transitions are induced in the lipid/polymer vesicles are correlated with the degree of lipid interactions and bilayer penetration of the tested compounds. The colorimetric platform could distinguish between three primary types of membrane-permeation profiles: bilayer-surface attachment, membrane penetration, and absence of lipid interactions. Application of complementary bioanalytical techniques corroborated the interpretation of the colorimetric data. Different pharmaceutical compounds were tested by the new assay. The results indicated clearly distinct membrane interaction profiles for molecules expected by conventional methods to have similar membrane-insertion properties (i.e., close log *D*/log *P* values). In addition, the new colorimetric assay pointed to *similar* membrane activities for molecules having highly divergent log *D*s.
Conclusions. The colorimetric assay facilitates "color coding" that could distinguish among different membrane permeation profiles. The data point to the usefulness of the platform for characterization of drug compound interactions with lipid assemblies. The new colorimetric technology constitutes a generic, extremely fast, and easily applicable approach for predicting and screening interactions of pharmaceutical compounds with lipid barriers.

KEY WORDS: blood–brain barrier; drug adsorption; membrane penetration; membrane transport; polydiacetylene.

INTRODUCTION

An essential task in identifying drug candidates is the assessment (and modification) of the passage of the examined molecules through physiological barriers such as the blood–brain barrier, the gastrointestinal tract, or the stratum corneum. Indeed, in many instances failure of promising pharmacological candidates as viable drugs can be traced to difficulties in transport of the compounds across those membranes. Current approaches for predicting permeation through lipid barriers rely on highly generic physicochemical parameters such as the partition coefficients between water and alcohol [so-called log *P*/log *D* values (1)], employ time-consuming chromatography methods (2) or artificial membranes (3), or utilize experimentally demanding methods based on live cell arrays (4). In many cases, however, the information obtained through application of such methods is ambiguous and cell type dependent. In particular, difficulties are encountered when trying to distinguish between transcellular passive diffusion and active, carrier-mediated transport (3,5).

The goal of this work is to develop and demonstrate a new technology for rapid screening of lipid interaction profiles of pharmacological compounds through application of a simple colorimetric assay. The colorimetric platform we have developed comprises an aqueous solution of mixed bilayer vesicles containing phospholipids [such as dimyristoylphosphatidylcholine (DMPC), used herein] and polydiacetylene (PDA), shown schematically in Fig. 1. Specifically,

[1] Ilse Katz Center for Meso- and Nano-Scale Science and Technology, Ben Gurion University, Beer Sheva 84105, Israel.
[2] Department of Chemistry, Ben Gurion University, Beer Sheva 84105, Israel.
[3] To whom correspondence should be addressed. e-mail: razj@bgu.ac.il
ABBREVIATIONS: DMPC, dimyristoylphosphatidylcholine, DMPS, dimyristoylphosphatidylserine; DSC, differential scanning calorimetry; NBD-PE, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)1,2-dihexadecanoyl-sn-glycero-3-phospo-ethanolamin, PDA, polydiacetylene; TFE, trifluoroethanol.

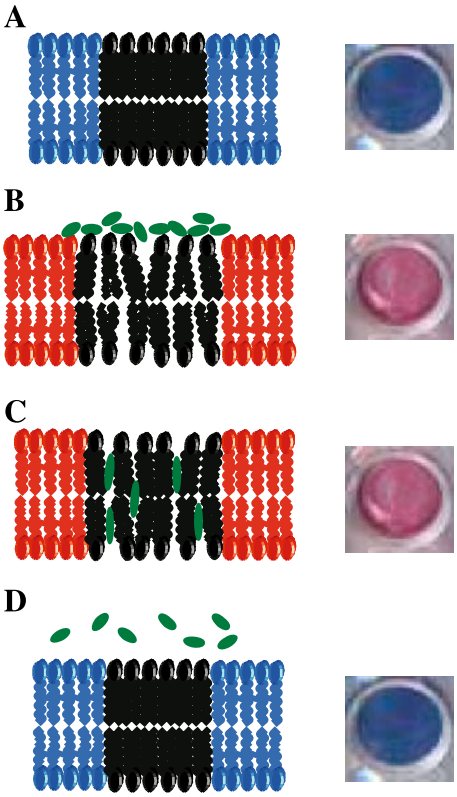

Fig. 1. Schematic descriptions of a section of the phospholipid/PDA bilayers and the chromatic transformations. (A) Initially prepared blue DMPC/PDA bilayers. The DMPC molecules are shown in black, PDA in blue; (B) blue–red transition induced by compounds bound at the bilayer surface; (C) blue–red transition induced by compounds penetrating into the phospholipid bilayer; (D) no color change is induced by molecules that do not interact with the bilayer.

the DMPC domains interspersed within the PDA matrix serve as the biomimetic membrane layer, while the polymer acts as a colorimetric reporter.

PDA exhibits unique chromatic properties. The initially polymerized PDA appears intense blue due to the conjugated (ene–yne) polymer backbone (6) (Fig. 1A). Previous studies have demonstrated that PDA vesicles and thin films undergo distinct blue–red colorimetric changes due to disruption in the delocalized electronic network induced by external structural perturbations (6,7). The chromatic transformations of PDA have been also observed in biological contexts: recent studies have demonstrated that the blue–red transitions of PDA could be induced in biomimetic assemblies of the polymer and biological constituents such as phospholipid bilayers by processes occurring exclusively within the bilayer domains (8–11). Here we show that DMPC/PDA vesicles could be employed as a useful platform for rapid screening of membrane interactions and bilayer penetration by pharmaceutical compounds.

MATERIALS AND METHODS

Materials

DMPC was purchased from Sigma. The diacetylenic monomer 10,12-tricosadiynoic acid was purchased from GFS Chemicals (Powell, OH, USA), washed in chloroform, and passed through a 0.45-μm filter before use.

Tris(hydroxymethyl)aminomethane (Tris-base buffer, $C_4H_{11}NO_3$), 2,2,2-trifluoroethanol (TFE), and the various pharmaceutical standards were purchased from Sigma. *N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (NBD-PE) was purchased from Molecular Probes, Inc. (Eugene, OR, USA). Sodium dithionite ($Na_2S_4O_2$) was purchased from Aldrich.

Vesicle Preparation

Preparation of vesicles containing lipid components (DMPC) and PDA (2:3 ratio) was carried out as the following: the constituents were dissolved in chloroform/ethanol (1:1) and dried together *in vacuo* up to constant weight, followed by addition of deionized water to final concentration of 1 mM, and probe-sonicated at 70°C for 3–4 min. The vesicle solution was cooled to room temperature and kept at 4°C overnight. The vesicles were then polymerized using irradiation at 254 nm for 30–40 s, with the resulting solutions exhibiting an intense blue appearance.

UV–Vis Measurements

Samples were prepared by adding different compounds to 0.06 mL vesicle solutions at 0.5 mM total lipid concentration and 25 mM Tris-base (pH 8). UV–vis spectroscopy measurements were carried out at 27°C and repeated three times on each sample on a Jena Analytical ELISA reader, using a 96-well microplate.

A quantitative value for the extent of the blue-to-red color transitions within the vesicle solutions is given by the colorimetric response (%CR), which is defined as follows (9):

$$\%CR = [(PB_0 - PB_I)/PB_0] \times 100$$

where

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

A is the absorbance either at the "blue" component in the UV–vis spectrum (640 nm) or at the "red" component (500 nm). (Note: "blue" and "red" refer to the visual appearance of the material, not its actual absorbance). PB_0 is the blue/red ratio of the control sample (before adding of compounds), and PB_I is the value obtained for the vesicle solution after adding of compounds.

Fluorescence Quenching

NBD-PE was dissolved in chloroform, added to the phospholipids and tricosadiynoic acid at 1 mol %, and dried together *in vacuo* before sonication (see "Vesicle Preparation"). Addition of NBD-PE did not affect the initial blue color of the vesicles or the subsequent color transitions. Samples for the fluorescence experiments were prepared by adding the pharmacological compounds at their respective EC_{50} concentrations to vesicle solutions at 0.5 mM (total lipid) concentration and 25 mM Tris-base (pH 8). The quenching reaction was initiated by adding sodium dithionite

from a 0.6 M stock solution prepared in 50 mM Tris-base (pH 11) buffer, to a final concentration of 10 mM. The decrease in fluorescence was recorded during 300 s at 28°C using excitation at 467 nm and emission at 535 nm on an Edinburgh FL920 spectrofluorimeter. The fluorescence decay was calculated as a percentage of the initial fluorescence measured before addition of dithionite.

Small-Angle X-Ray Scattering

Nonsonicated multilamellar suspensions (300 mM total lipids) were prepared by dissolving the dry lipid and diacetylene constituents in distilled water. Samples were inserted into 1.5-mm-diameter thin-wall quartz X-ray capillaries. Scattering experiments were performed at room temperature using Ni-filtered Cu radiation (0.154 nm) from an Elliott GX6 rotating anode X-ray generator operating at a power rating of 1.2 kW. The X-radiation was further monochromated and collimated by a 20-cm Franks mirror and a series of slits and height limiters. The scattering was measured by a linear position-sensitive detector of the delay-line type and histogrammed into 256 channels with a Z80 microprocessor. The camera parameters were calibrated using anhydrous cholesterol. Typical exposure time was 1 h.

Differential Scanning Calorimetry

Vesicle concentrations used in the experiments were 2 mM. Samples were prepared by adding the pharmacological compounds at their respective EC_{50} concentrations to the vesicle solutions. The differential scanning calorimetry (DSC) experiments were performed on a VP-DSC (MicroCal, USA). Distilled water served as a blank. Heating scans were run at a rate of 1.5°C/min. Data analysis was performed using the software provided by the Microcal Origin 6.0.

RESULTS

The basic thrust of the colorimetric assay as a tool for characterizing membrane interactions is depicted schematically in Fig. 1. The primary factor responsible for the color transitions of PDA has been ascribed to structural perturbations of the pendant side chains of the polymerized network (6,7). Accordingly, color transformation can be induced through binding of the tested compounds at the lipid–water interface (Fig. 1B) or through penetration into the lipid bilayer (Fig. 1C). Molecules that do not interact with the lipid layers would not change the initial blue color of the vesicles (Fig. 1D). The experiments we performed were designed to demonstrate the applicability of the colorimetric assay for screening lipid interactions of a large data set of common pharmacological standards and in particular to distinguish among the different lipid permeation scenarios, schematically depicted in Fig. 1.

To investigate whether the colorimetric assay can differentiate among the three types of bilayer interaction profiles (Fig. 1), we recorded the colorimetric (%CR, see "Materials and Methods") dose–response curves of different molecules added to the DMPC/PDA vesicle suspensions. Dose–response curves of representative compounds are depicted in Fig. 2. The molecules displayed in Fig. 2 have been previously studied both *in vivo* and *in vitro*, and biophysical models for their membrane interactions and passage were proposed in the literature. The color changes induced by the different pharmaceutical compounds essentially occurred within several seconds after mixing with the vesicles and remained almost constant for long periods after (approaching hours). All measurements reported here were carried out 60 s after addition of compounds to the vesicles (a representative time-dependent colorimetric response is shown in the Electronic supplementary material is available for this article at http://dx.doi.org/10.1007/s11095-006-9569-1 and is accessible for authorized users).

Figure 2 shows that the %CR dose–response profiles could be divided into three subgroups showing significantly divergent colorimetric properties. Specifically, the compounds exhibit differences both in extent of color change they induced (the %CR values recorded), as well as in the concentration ranges in which the blue–red transitions occurred. Nortiptyline, imipramine, and maprotiline (Fig. 2, I) featured sigmoidal dose–response curves in which the maximal red color (highest %CR) was reached at concentrations smaller than

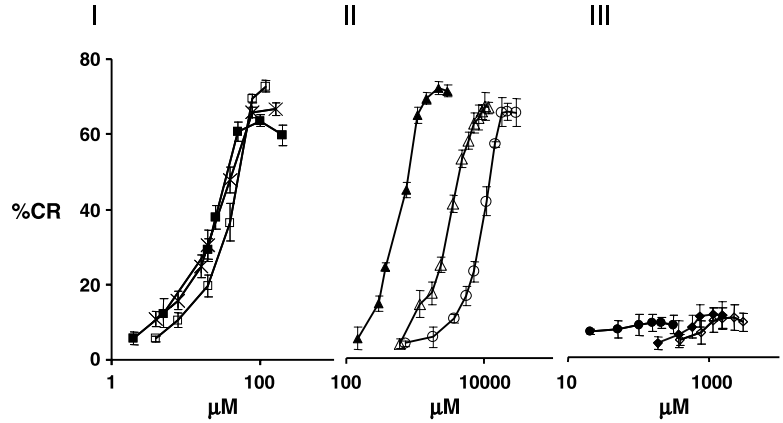

Fig. 2. Colorimetric dose–response curves of selected pharmacological standards. The compounds were divided into three subgroups according to their colorimetric responses (see text): I, nortiptyline (■), imipramine (□), maprotiline (×); II, metoprolol (▲), acebutolol (△), valproic acid (○); III, diclofenac (♦), amoxicillin (●), procainamide (◇).

100 μM. Metoprolol, acebutolol, and valproic acid (Fig. 2, II) gave rise to the blue–red transformations at orders of magnitude higher concentrations of between 100 and 10,000 μM (Fig. 2B), whereas diclofenac, procainamide, and amoxicillin (Fig. 2, III) did not induce noticeable color changes (i.e., very small %CR values) even at high concentrations. The assignment of the three colorimetric profiles to the three structural models depicted in Fig. 1 (groups I, II, III) is discussed in detail in the "Discussion" section.

To characterize the assemblies formed through interaction of the pharmaceutical compounds with the DMPC/PDA vesicles and to relate the colorimetric data to the membrane interaction models shown in Fig. 1, we applied several analytical methods (Fig. 3). The bioanalytical techniques employed in the experiments, including fluorescence quenching (Fig. 3A), small-angle X-ray scattering (SAXS, Fig. 3B), and DSC (Fig. 3C), have been previously used in diverse studies for analysis of bilayer interactions and binding.

Figure 3A presents the results of a fluorescence quenching experiment utilizing phospholipid/PDA vesicles additionally incorporating the phospholipid derivative NBD-PE. This experiment examines the extent of lipid-surface perturbation through measurement of the quenching of the NBD dye fluorescence emission by water-soluble sodium dithionite (12). Because the fluorescent NBD moieties in the vesicles are exposed at the head group region of the phospholipid bilayers, the experiment depicted in Fig. 3A essentially probes molecular interactions and perturbations occurring at the vesicle

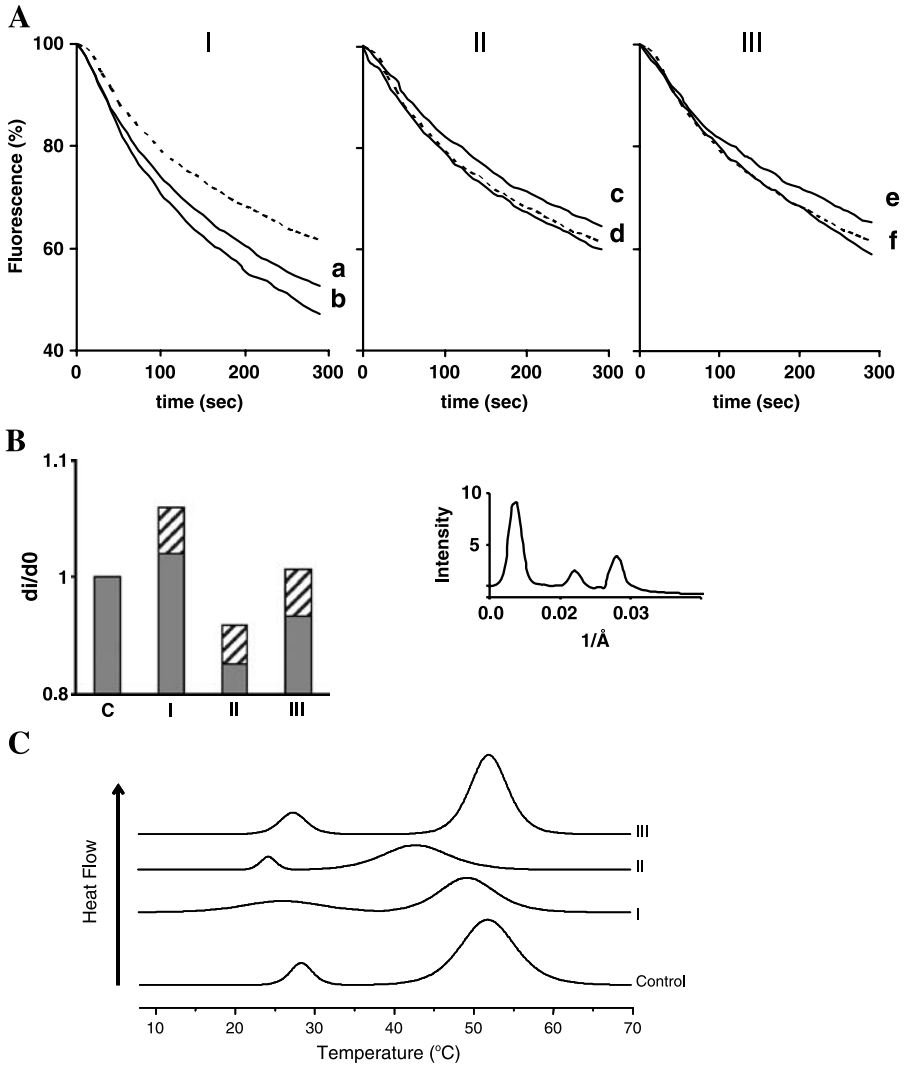

Fig. 3. Spectroscopic analysis. (A) Quenching of the fluorescence emission (535 nm) of NBD-PE incorporated within the DMPC/PDA vesicles by soluble dithionite. Broken curves, control sample (vesicles without addition of pharmacological compounds). a, maprotiline; b, nortiptyline; c, metoprolol; d, acebutolol; e, amoxicillin; f, procainamide. (B) Cumulative SAXS data. Striped sections of the bars indicate the range of interbilayer spacings recorded. Leftmost bar, control vesicles (no added compounds); I, compounds from subgroup I added; II, compounds from subgroup II added; III, compounds from subgroup III added. (C) Representative DSC traces. Control, vesicles with no added compounds; I, nortiptyline added to vesicles; II, metoprolol added; III, procainamide added.

Table I. Colorimetric Data Recorded for Different Pharmacological Standards

	Compound	MW	log *D* (pH 8)	EC_{50} (μM)	CR (%)
Group I					
1	Amitriptyline hydrochloride	313.9	4.89	25	34
2	Desipramine hydrochloride	302.8	3.61	28	36
3	Imipramine hydrochloride	316.9	2.97	39	36
4	Maprotiline hydrochloride	313.9	2.00	24	31
5	Nortriptyline hydrochloride	299.8	3.61	22	32
6	Perphenazine	404.0	4.40	22	39
7	Promethazine hydrochloride	320.9	3.66	26	34
8	Propafenone hydrochloride	377.9	3.31	80	30
9	DL-Propranolol hydrochloride	295.8	1.93	92	34
10	Terfenadine	471.7	5.32	36	29
11	Tetracaine	264.4	3.05	56	32
12	Quinidine hydrochloride	378.9	2.29	100	31
Group II					
13	Acebutolol hydrochloride	372.9	1.45	2900	31
14	BAPTA-AM	764.7	4.62	1600	34
15	Diazepam	284.7	3.86	260	27
16	DP-109	832.4		540	34
17	Lidocaine	234.3	1.72	390	30
18	Metoprolol tartrate salt	684.8	0.59	560	34
19	Valproic acid sodium salt	166.2	−0.42	10000	31
Group III					
20	Amoxicillin	365.4	−2.88	16	4
21	Benzocaine	165.2	2.49	340	3
22	Carbamazepine	236.6	3.05	130	4
23	Chloramphenicol	323.1	1.02	50	6
24	Coumarin	146.1	1.39	170	9
25	Dexamethasone	392.5	2.06	70	1
26	Diclofenac Sodium Salt	318.1	−0.35	200	1
27	Digoxin	780.9	1.14	420	4
28	Estradiol	272.4	4.13	400	8
29	Hydrocortisone	362.5	1.43	440	10
30	Ibuprofen sodium salt	228.3	0.25	620	12
31	Indapamide	365.8	2.07	20	3
32	Indomethacin	357.8	−0.53	20	3
33	Naproxen	230.3	−0.48	10	5
34	Procaine hydrochloride	272.8	1.65	350	5
35	Procainamide hydrochloride	271.8	−0.61	550	5
36	Theophylline anhydrous	180.2	−0.05	250	3
37	Thymidine	242.2	−4.07	300	3

surface (13). The fluorescence data shown in Fig. 3A demonstrate a clear difference between the increase in quenching (compared to the control sample in which only dithionite was added to the NBD-PE/DMPC/PDA vesicles, broken curve) induced by compounds ascribed to subgroup I (nortiptyline and imipramine, Fig. 3A, I) and the other compounds yielding the color profiles of group II (metoprolol and acetobutolol, Fig. 3A, II) and group III (procainamide and diclofenac, Fig. 3A, III), which exhibited similar quenching as the control.

Figure 3B shows the cumulative SAXS data for the nine compounds depicted in Fig. 2, in comparison to the control DMPC/PDA vesicles. SAXS analysis facilitates investigation of the width and ordering of lipid layers (14). Previous SAXS investigation characterized phospholipid/PDA vesicles and bilayer modifications induced through interactions of the vesicles with different molecules (15). The inset in Fig. 3B features the SAXS spectrum of the control DMPC/PDA vesicles, exhibiting peaks corresponding to the organized PDA bilayers (reflection at around 0.023 $Å^1$), and the phase-separated DMPC bilayers (reflections at approximately 0.014 and 0.028 $Å^1$) (15).

The bar diagram in Fig. 3B shows the modification of the main bilayer spacing of DMPC (peak at around 0.014 $Å^1$) following addition of the compounds producing the colorimetric data in Fig. 2. More importantly, the PDA reflection (at 0.023 $Å^1$, inset) was hardly affected by addition of the molecules investigated (data not shown), indicating minimal interaction with the polymer matrix. The striped areas within the bars in Fig. 3B correspond to the ranges of DMPC bilayer spacings recorded in the SAXS experiments of the different compounds. Indeed, the SAXS results demonstrate that distinct effects occur following addition of the three compound subgroups. Specifically, following addition of compounds belonging to subgroup I (nortiptyline, imipramine, and maprotiline) the bilayer spacings increase (Fig. 3B). However, the interbilayer spacings decrease following interactions of the vesicles with group II (metoprolol, acebutolol, and valproic

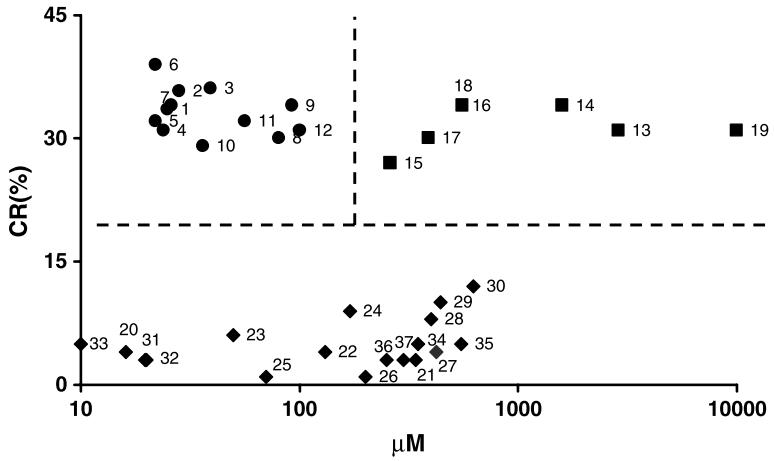

Fig. 4. Colorimetric EC_{50} values recorded at the respective concentrations in DMPC/PDA vesicle solutions for different compounds. Numbering corresponds to the compounds in Table I. Subgroup I (●), subgroup II (■), subgroup III (♦).

acid), whereas substances belonging to group III (diclofenac, procainamide and amoxicillin) do not significantly alter the bilayer ordering (Fig. 3B).

DSC analysis (Fig. 3C) complemented the fluorescence and SAXS characterization and facilitates evaluation of the effects of the interacting compounds on the organization and cooperativity properties of both lipid and polymer domains within the vesicles (16). Fig. 3C depicts the thermotropic phase behavior of the DMPC/PDA vesicles, specifically the consequences of adding nortiptyline (group I), metoprolol (group II), or procainamide (group III). The DSC thermogram of the control DMPC/PDA vesicle suspension (Fig. 3C) exhibits the typical highly cooperative phase transition of DMPC (at approximately 28°C) and PDA (around 53°C) (15).

The DSC thermograms recorded after addition of nortiptyline (group I) or metoprolol (group II) indicate that each induced different modifications of temperatures as well as cooperativity of the phase transitions (Fig. 3C). Specifically, amytriptiline shifted the peaks of the transition temperatures of DMPC and PDA to lower temperatures (Fig. 3C, I) and, perhaps more significantly, gave rise to extreme broadening of the phase transition, indicating substantial reduction of molecular ordering within the DMPC and PDA domains (16). The overall enthalpies of the transitions (1130 ± 20 and 6560 ± 80 kcal/mol for DMPC and PDA, respectively, calculated from the areas under the peaks) were not significantly reduced following nortiptyline addition, indicating that most of the phospholipid and polymer moieties within the vesicles still retained cooperative behavior even after nortiptyline binding.

Similar to nortiptyline, metoprolol altered the DSC trace (Fig. 3C, II); the thermotropic effects, however, were different for the two compounds. Specifically, metoprolol gave rise to large temperature shifts for both DMPC and PDA transitions. Importantly, the enthalpy of the DMPC phase transition at around 25°C was reduced by approximately 70% (to 380 ± 10 kcal/mol) after addition of metoprolol (calculated areas under the DSC transition, Fig. 3C, II). This result indicates significant disruption of the DMPC assemblies and their cooperative phase transition induced by metoprolol. In contrast to the considerable effects of both nortiptyline and metoprolol upon the thermotropic transitions of DMPC, procaineamide (group III) hardly affected the DSC trace of the DMPC/PDA vesicles (Fig. 3C). The latter observation echoes the insignificant structural effects induced by compounds belonging to group III—apparent both in the fluorescence quenching experiments (Fig. 3A) and in the SAXS analysis (Fig. 3B). Fluorescence quenching and DSC experiments using other pharmaceutical compounds (summarized in Table I) gave qualitatively similar results with regard to the effects on the polymer and phospholipid organizations.

To determine the general applicability of the colorimetric vesicle system we assayed a large set of common pharmaceutical standards and drug compounds (Fig. 4 and Table I). Figure 4 depicts the colorimetric EC_{50} values, shown in logarithmic concentration scale, extracted from dose–response curves similar to the ones shown in Fig. 2.

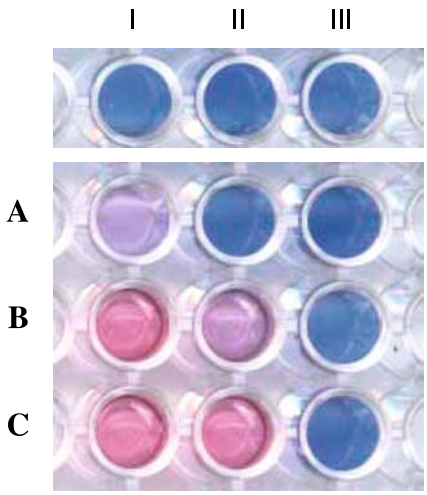

Fig. 5. Scanned section of a 96-well plate containing 1 mM DMPC/PDA vesicles to which were added I, nortiptyline; II, metoprolol; III, procainamide. The concentrations used were (A) 10 μM, (B) 1 mM, (C) 1 M. The top row depicts the initial blue vesicle solutions before addition of compounds.

Each data point in Fig. 4 corresponds to an individual molecule tested (numerically presented in Table I). The distribution of data points in Fig. 4 echoes the division of tested compounds into three groups; as depicted in Fig. 2, each exhibits typical %CR values and concentration ranges. Specifically, Fig. 4 identifies compounds for which the EC_{50} values were in the tens of micromolar concentration range (circles), molecules exhibiting EC_{50} at a much higher, millimolar to molar concentration range (squares), and substances that do not induce significant blue–red transitions (diamonds).

DISCUSSION

The experimental data in Figs. 2–4 demonstrate that DMPC/PDA vesicles emitted visible colorimetric signals upon interactions with pharmacological compounds, and that significant differences were observed among the colorimetric profiles of the molecules. The choice of constructing vesicles with DMPC as the phospholipid component was due to the fact that this molecule is present in varied mammalian membranes and has probably been the most abundantly used phospholipid in numerous membrane model studies. More importantly, experiments carried out using polymerized vesicles containing completely different lipid compositions, such as DMPC/sphingomyelin/dimyristoylphosphatidylserine (DMPS), or even vesicles incorporating total lipid extracts from cell lines such as CACO-2 (See supplementary materials), gave similar chromatic profiles compared to the DMPC/PDA, confirming the validity of the DMPC/PDA model for studying membrane penetration.

The molecules tested could be distinguished according to the colorimetric response elicited following their addition to the DMPC/PDA vesicles, overall summarized in Fig. 4. Several compounds induced maximal colorimetric response at concentrations that were lower than 100 μM (denoted group I in Fig. 2, shown as circles in Fig. 4). Other compounds gave rise to color changes at a higher concentration range of 100 μM–M (group II in Fig. 2, squares in Fig. 4), whereas other screened molecules did not induce noticeable color transitions (group III in Fig. 2 and diamonds in Fig. 4).

The colorimetric results presented in Fig. 2 can be analyzed according to the structural model schematically depicted in Fig. 1, which is based on the known mechanisms for color transitions in PDA systems. Specifically, the colorimetric transformations in PDA assemblies occur through creation of "structural defects" in the conjugate network of the polymer. Such disruptions have been related to structural alterations of the pendant side chains of the polymer at the vesicle interface, thereby inducing the blue–red color change (6,7). Previous analyses of color transitions induced in phospholipid/PDA systems verified that surface perturbations are the predominant factor for induction of the PDA color changes (17). According to this description, molecules that preferably aggregate at the lipid/water interface (rather than penetrate or cross through the lipid barrier) would give rise to pronounced surface perturbations, thus inducing color changes even at very low concentrations (Figs. 2, I and 4). However, compounds that tend to insert into the bilayer would give rise to smaller surface perturbations, leading to higher concentrations inducing blue–red transitions, as indeed shown in Figs. 2, II and 4. Similar relationships between the degree of color changes in phospholipid/PDA vesicles and the extent of bilayer penetration were observed for membrane-active peptides (10,18).

Application of several analytical techniques supported the interpretation of the colorimetric data according to the different structural models. The fluorescence quenching experiments depicted in Fig. 3A provided information on the extent of interactions and perturbations occurring at the phospholipids' head group region (i.e., the vesicle surface). In such experiments, quenching of the fluorescence of the NBD probe (covalently attached in proximity of the phospholipid head groups) by soluble dithionite is monitored. Faster fluorescence quenching is indicative of surface perturbations leading to exposure of the NBD moieties to the dithionite in the aqueous solution (19). Indeed, the rapid fluorescence quenching observed when either nortiptyline or imipramine (group I, Fig. 3A) was added to the NBD-PE/DMPC/PDA vesicles is ascribed to the significant surface interactions of these compounds according to the structural model in Fig. 1B. The relative absence of quenching for the other compounds shown in Fig. 3A is most likely explained by the insertion into the bilayer core rather than surface interactions (in case of metoprolol and acebutolol, Fig. 3A, II), or by the absence of bilayer interactions (procainamide and amoxicillin, Fig. 3A, III).

SAXS analysis (Fig. 3B) further illuminated the relationships between the different colorimetric profiles and the structural aspects of the complexes formed between the compounds and the phospholipid/PDA vesicles. The bar diagram in Fig. 3B indicates that the interbilayer spacing of DMPC underwent experimentally significant increase following binding of molecules belonging to group I (Fig. 3B, I). This result is fully consistent with surface localization of the compounds (Fig. 1B)—thus leading to greater distances between adjacent bilayers within the DMPC/PDA multilamellae (20).

An opposite effect on the DMPC reflections was detected following addition of group II compounds to the DMPC/PDA vesicles (Fig. 3B, II). The smaller interbilayer spacing for DMPC in this case is most likely due to the insertion of the compounds into the bilayer core according to the structural model shown in Fig. 1C, resulting in bilayer contraction. This result is similar to previously observed bilayer narrowing induced by transmembrane peptides (10,18). No structural effects on the DMPC bilayers were recorded following addition of group III compounds to the chromatic vesicles—consistent with the assignment of group III to bilayer-inactive species.

The DSC analysis (Fig. 3C) yielded additional insight into the association of the pharmacological standards with the DMPC/PDA vesicles and further highlighted the correlation between the colorimetric assay results and the structural models. In particular, the DSC thermograms exposed the effects of added compounds upon the organization and cooperative properties of the phospholipid domains. Fig. 3C demonstrated that both nortiptyline (group I) and metoprolol (group II) decreased the molecular ordering of PDA and DMPC. Notably, significant differences in the effects of compound addition on the enthalpy of the DMPC

transition were detected. Whereas nortiptyline addition did not diminish the overall enthalpy (Fig. 3C, I), metoprolol induced almost 70% reduction of the enthalpy (reflected in the smaller area under the DMPC transition, Fig. 3C, II).

The pronounced difference in the enthalpy effect between nortiptyline and metoprolol is consistent with the structural models ascribed to these compounds (Fig. 1). Surface binding of nortiptyline (Fig. 1B) most likely affected the ordering of the DMPC molecules within the vesicle bilayers; however, such surface interactions did not alter the overall domain organization of the lipids (and consequently the enthalpy was retained, Fig. 3C, I). In contrast, metoprolol penetration into the phospholipid bilayer (Fig. 1C) would significantly disrupt the lipid organization and consequently diminish the cooperative phase transitions, as indeed apparent in Fig. 3C, II. The DSC experiment also confirms the absence of membrane interactions of procaine (Fig. 3C), echoing similar data in the fluorescence (Fig. 3A) and SAXS (Fig. 3B) analyses.

The generality of the colorimetric assay as a tool for screening membrane interactions of pharmacological compounds is depicted in Fig. 4 and Table I. The graph in Fig. 4 clearly shows that the examined molecules can be assigned to one of the structural models according to their colorimetric properties. Inspection of the log *D* (defined as the water/alcohol partition coefficient (log *P*) at a distinct pH value—here pH 8.0) and the colorimetric EC_{50} values of the various compounds summarized in Table I shows that the commonly used log *D* values do not predict lipid interaction characteristics as outlined by the colorimetric assay. In particular, the colorimetric assay pointed to different bilayer permeation profiles among molecules with almost identical log *D* values. For example digoxin (log $D = 1.93$) is ascribed by the colorimetric assay to group III, whereas lidocaine (log $D = 1.72$) is assigned to group II. Previous studies indeed identified significant differences in the biological properties and membrane permeation of digoxin and lidocaine (21,22). The colorimetric assay is furthermore capable of identifying similar lipid interactions in molecules having significantly different log *D* values. For example, amitriptyline with log *D* of 4.88 and maprotiline having log *D* of 1.88 are both predicted, according to the colorimetric assay, to exhibit high lipid-surface affinity (group I in Table I). Biological studies are again consistent with this prediction (22,23). The comparative analysis depicted in Fig. 4 emphasizes the utilization of the new colorimetric assay as an independent predictive tool for lipid binding and permeation of pharmaceutical substances.

More importantly, the assignment of compounds to group III does not necessarily imply that such molecules cannot pass through lipid membranes. Rather, this means that such molecules cannot undergo passive transport (passive diffusion) through the bilayer. Possibilities exist, of course, that compounds belonging to group III could pass through lipid bilayers via specific transporters or receptors (active transport). Experiments are under way in our laboratory to exploit the colorimetric assay for evaluating active transport phenomena by incorporating receptors/transporters within the chromatic vesicles.

Figure 5 visually summarizes the practical utilization of the colorimetric assay for rapid screening of membrane interactions of pharmacological molecules. Each tested compound would be examined in three concentrations: single micromolar range (row A in Fig. 5), millimolar range (row B), and molar range (row C). The produced blue–red color coding would be predictive as to the type of membrane interaction properties of the examined compound. For example, a surface-attached molecule (column I in Fig. 5) would induce a purple (or red) color at 50 μM (A), and maximal red colors at the higher concentrations employed (B, C). The corresponding color code for a bilayer-penetrating compound (column II in Fig. 5) would be blue/purple/red (Fig. 5, middle column), whereas a substance belonging to the third group (nonmembrane active compounds) would produce blue/blue/blue (or blue/blue/purple), respectively, at the three concentrations examined (Fig. 5, column III).

The phospholipid/PDA assay exhibits important practical advantages for application as a generic tool for drug screening. The vesicle solutions can be placed and stored for long periods in conventional 96-well (or 384-well) plates. The colorimetric transitions are induced within a very short time (seconds) after mixing the reagents. The new technique is robust and easy to apply, and data for large compound libraries can be obtained in a few minutes. The colorimetric assay can thus become a highly useful tool for predicting membrane interactions and bilayer permeation at early stages of drug development and profiling.

ACKNOWLEDGMENT

Generous financial assistance from the Israel Ministry of Trade and Industry through the MAGNET Program, Consortium Pharmalogica, is acknowledged.

REFERENCES

1. I. Tetko and P. Bruneau. Application of ALOPGPS to predict 1-octanol/water distribution coefficients, logP, and logD, of AstraZeneca in-house database. *J. Pharm. Sci.* **93**:3103–3110 (2004).
2. L. Escuder-Gilabert, M. Molero-Monfort, R. M. Villanueva-Camanas, S. Sagrado, and M. J. Medina-Hernandez. Potential of biopartitioning micellar chromatography as an *in vitro* technique for predicting drug penetration across the blood–brain barrier. *J. Chromatogr.* **807**:193–201 (2004).
3. P. Garberg, M. Ball, N. Borg, R. Cecchelli, L. Fenart, R. Hurst, T. Lindmark, A. Mabondzo, J. Nilsson, T. Raub, D. Stanimirovic, T. Terasaki, J. Oberg, and T. Osterberg. *In vitro* models for the blood–brain barrier. *Toxicol. In Vitro* **19**:299–334 (2005).
4. C. Masungi, C. Borremans, B. Willems, J. Mensch, A. Van Dijck, P. Auguctijns, M. Brewster, and M. Noppe. Usefulness of a novel caco2 cell perfusion system. I. *In vitro* prediction of the absorption potential of passively diffused compounds. *J. Pharm. Sci.* **93**: 2507–2521 (2004).
5. C. Wu and L. Benet. Predicting drug disposition via application of BCS: transport/absorption/elimination interplay and development of a biopharmaceutics drug disposition classification system. *Pharm. Res.* **22**:11–23 (2005).
6. H. Ringsdorf, B. Schlarb, and J. Venzmer. Molecular architecture and function of polymeric oriented systems: models for the study of organization, surface recognition, and dynamics of biomembranes. *Angew. Chem. Int. Ed. Engl.* **27**:113–158 (1988).
7. S. Okada, S. Peng, W. Spevak, and D. Charych. Color and chromism of polydiacetylene vesicles. *Acc. Chem. Res.* **31**:229–239 (1998).

8. D. H. Charych, J. O. Nagy, W. Spevak, and M. D. Bednarski. Direct colorimetric detection of a receptor–ligand interaction by a polymerized bilayer assembly. *Science* **261**:585–588 (1993).
9. S. Kolusheva, L. Boyer, and R. Jelinek. A colorimetric assay for rapid screening of antimicrobial peptides. *Nat. Biotechnol.* **18**: 225–227 (2000).
10. S. Kolusheva T. Shahal, and R. Jelinek. Peptide–membrane interactions studied by a new phospholipid/polydiacetylene colorimetric vesicle assay. *Biochemistry* **39**:15851–15859 (2000).
11. S. Y. Okada, R. Jelinek, and D. Charych. Induced color change of conjugated polymeric vesicles by interfacial catalysis of phospholipase a2. *Angew. Chem. Int. Ed.* **38**:655–659 (1999).
12. A. Chattopadhyay and E. London. Parallax method for direct measurement of membrane penetration depth utilizing fluorescence quenching by spin-labeled phospholipids. *Biochemistry* **26**:39–45 (1987).
13. J. C. McIntyre and R. G. Sleight. Fluorescence assay for phospholipid membrane asymmetry. *Biochemistry* **30**:11819–11827 (1991).
14. S. W. Hui and N.-B. He. Molecular organization in cholesterol–lecithin bilayers by x-ray and electron diffraction measurements. *Biochemistry* **22**:1159–1164 (1983).
15. S. Kolusheva, E. Wachtel, and R. Jelinek. Biomimetic lipid/polymer colorimetric membranes: molecular and cooperative properties. *J. Lipid Res.* **44**:65–71 (2003).
16. D. Papahadjopoulos. Effects of bivalent cations and proteins on thermotropic properties of phospholipid membranes. Implications for the molecular mechanism of fusion and endocytosis. *J. Colloid Interface Sci.* **58**:459–470 (1977).
17. R. Volinsky, S. Kolusheva, A. Berman, and R. Jelinek. Microscopic visualization of alamethicin incorporation intos model membrane monolayers. *Langmuir* **20**:11084–11091 (2004).
18. D. P. Satchell, T. Sheynis, Y. Shirafuji, S. Kolusheva, A. Ouellette, and R. Jelinek. Interactions of mouse Paneth cell-defensins with membranes studied by a biomimetic lipid/polymer colorimetric assay. *J. Biol. Chem.* **278**:13838–13846 (2003).
19. M. Katz, H. Tsubery, S. Kolusheva, A. Shames, M. Fridkin, and R. Jelinek. Lipid binding and membrane penetration of polymyxin B derivatives studied in a biomimetic vesicle system. *Biochem. J.* **375**:405–413 (2003).
20. Y. Kobayashi and K. Fukada. Characterization of swollen lamellar phase of dimyristoylphosphatidylcholine–gramicidin a mixed membranes by DSC, SAXS, and densimetry. *Biochim. Biophys. Acta* **1371**:363–370 (1998).
21. L. Fraceto, L. Pinto, L. Franzoni, A. Braga, A. Spisni, S. Schreier, and E. de Paula. Spectroscopic evidence for a preferential location of lidocaine inside phospholipid bilayers. *Biophys. Chem.* **99**: 229–243 (2002).
22. P. Suomalainen, C. Johans, T. Soederlund, and P. Kinnunen. Activity profiling of drugs applied to the prediction of blood–brain barrier permeability. *J. Med. Chem.* **47**:1783–1788 (2004).
23. Y. Sudoh, E. Cahoon, P. Gerner, and G. Wang. Tricyclic antidepressants as long-acting local anesthetics. *Pain.* **103**:49–55 (2003).