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Spingomyelin Ability to Act as Chiral Selector using Nanodisc Electrokinetic Chromatography

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Abstract

Understanding how stereochemistry affects interactions with cell membranes is important for effective drug development. Chirality has been shown to greatly effect pharmaceutical distribution and metabolism within the cell. However it has been thought that interactions with, and passive diffusion through, the membrane are not stereochemically selective. Various studies have produced conflicting results regarding whether interactions with lipid bilayers are or can be stereoselective. In the current work, stereoselective interactions between a pair of atropisomers, R-(+)/(S)-(–) 1,1'-Bi-2-naphthol, and sphingomyelin nanodisc bilayers, are demonstrated. This is accomplished using nanodisc electrokinetic chromatography, demonstrating that this approach is sensitive to subtle differences in affinity between small molecule probes and lipid bilayers. Using the same approach, no evidence of stereoselectivity was observed using enantiomer or diastereomer probes of varied chemistry and structure.

Keywords

Lipid Bilayer; Stereoselectivity; Capillary electrophoresis; Atropisomer

1. Introduction

Understanding the biological mechanisms that determine the efficacy of chiral pharmaceuticals is important to drug development. Although chiral molecules contain the same molecular formulas and connectivity, they can have significantly different activity and toxicity. This is because stereochemistry can greatly affect a pharmaceutical's absorption, distribution, metabolism, and excretion^{1,2}. An atropisomer is a form of stereoisomer that results from sterically hindered rotation around one or more single bonds^{3,4}. Atropisomers can also have significant differences in activity; the (R)-(+)/(S)-(–) configurations of Telenzepine, a selective muscarinic antagonist with a stereogenic C-N axis used for the treatment of peptic ulcers,⁵ were found to have a 500-fold difference in activity⁴. As a result of these significant biological effects, most pharmaceuticals developed are achiral or are stereochemically pure^{6,7}.

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Due to the inherent cost of pharmaceutical development, it is important to have fast, efficient, and relatively inexpensive techniques to study drug absorption, distribution, metabolism, and excretion. Capillary electrophoresis (CE) is a separation and analysis technique, which allows for quick analysis, high theoretical plate counts and resolution using nanoliter sample volumes. CE has become a valuable technique for studying biomolecule affinity^{8–10}, and membrane characterization^{11–14} as well as pharmaceutical absorption^{15–17} and metabolism^{18–20}. We recently introduced nanodisc electrokinetic chromatography (NEKC), an adaptation of CE using lipid bilayer nanodiscs as additives, as a technique for the study of small molecule interactions with lipid bilayers^{13,14}. Using this technique it was demonstrated that solvation of small molecules into lipid bilayers was sensitive to slight structural changes in lipid head group chemistry¹⁴. The sensitivity of this approach to observe and characterize small differences in affinity for the lipid bilayer nanodiscs should render it sensitive to differences in affinities between stereoisomers.

There is significant disagreement among previous studies of the effects of lipid stereochemistry on bilayer properties and affinities. Some studies have shown that phospholipids showed no preferential interaction with either R or S chiral molecules and that the changing the stereocenters of the lipids did not affect bilayer physical properties^{21–23}. While more recent work has shown that after 24–48 hour incubation periods enantiomerically pure liposomes could preferentially absorb L-amino acids and preferentially absorb higher concentrations of one ibuprofen enantiomer over the other^{24,25}.

A pair of papers published in 2001 suggested that sphingomyelin bilayers could distinguish between *nat*-cholesterol and *ent*-cholesterol, its unnatural enantiomer^{26,27}. These findings were rebuked by the extensive analysis of Mannock *et al.* in 2003 who concluded that significant enantioselective cholesterol-sphingolipid interactions do not occur in model membrane systems²⁸. Although the properties of sphingomyelin have been studied extensively in the literature,^{29–32} there has not been extensive work on the interaction between atropisomers and sphingomyelin. Understanding if the stereochemistry of an atropisomer affects affinity for sphingomyelin has the potential for implications in drug development and in the study of membrane function.

Sphingomyelin, seen in Figure 1, is a class of sphingolipids that contain a phosphocholine head group, sphingosine base and the acyl group linked to the amide nitrogen³³. The sphingosine base contains two chiral centers with a D-*erythro* or 2S, 3R configuration, which differentiates it from other phosphocholine lipids, which only contain one chiral center. The stereochemistry of sphingomyelin has been shown to play an important role in its biophysical properties when enantiomerically pure sphingomyelin is compared to its racemate³⁴. Sphingomyelin is a major lipid component of cell membranes and studies have shown that sphingomyelin distribution in membranes of the aorta and arteries increases with age³³.

The conflicting results in the literature regarding the chiral selectivity of sphingomyelin lipid bilayers led to us to apply NEKC to characterize enantiomer, diastereomer and atropisomer interactions with these bilayers. In general, no detectable enantiomer or diastereomer selectivity was observed, but significant selectivity was observed in the separation of (R)-

(+)/(S)-(-)-1,1'-Bi-2-naphthol, an atropisomer shown in Figure 1. The successful separation demonstrates that NEKC is a sensitive technique for measuring small molecule bilayer interactions and provides tangible evidence of atropisomer selectivity in sphingomyelin bilayers.

2 Materials and Methods

2.1 Chemicals

Sodium phosphate monobasic monohydrate, sodium phosphate dibasic heptahydrate, potassium hydroxide, and hydrochloric acid were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Egg sphingomyelin and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Copolymer Xiran 25010 (Polyscope Polymers, Geleen, Netherlands), was a gift from Stefan Scheidelaar of Utrecht University. Analytes (R)-(+)-1,1'-Bi-2-naphthol and (S)-(-)-1,1'-Bi-2-naphthol used for analysis were obtained from Sigma-Aldrich (Saint Louis, MO, USA).

2.2 Preparation of Xiran 25010 copolymer

The Xiran 25010 copolymers use the same preparation that has been described in the literature^{35,36}. A 5% (w/v) solution of Xiran 25010 is suspended in a 1 M KOH solution and refluxed for 6 h. To confirm the reaction completion FTIR analysis is employed, monitoring the shift in the carbonyl signal from 1780 to 1560 cm⁻¹. The copolymer was precipitated using 6 M HCl to create a 1.1 M HCl solution. The precipitate was washed five times with 0.1 M HCl. The copolymer was freeze-dried and stored at -20°C.

2.3 Preparation of Nanodiscs

Sphingomyelin and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine nanodiscs were synthesized as follows. Lipids dissolved in chloroform were dried using a rotary evaporator and rehydrated to 5 mM lipid concentration using a 25 mM phosphate pH 7.0 buffer. Ten freeze-thaw cycles were performed on the lipid suspension using a dry ice-ethanol bath and a sonicator with a water temperature set to 45°C to form multilamellar vesicles. The Xiran 25010 copolymer suspended in 25 mM phosphate buffer is heated to 45°C to help solubilize the copolymer and then the solution is adjusted to pH 7.0. The copolymer was added to the multilamellar suspension contained a 2.00:1.00 (w:w) copolymer to lipid ratio. The solution was then lightly vortexed for 5 min before being placed in a heated bath for 30 minutes to complete nanodisc formation. Once the conversion is complete, the nanodiscs are placed in the refrigerator for storage.

2.4 EKC characterization

Phosphate buffers were prepared using sodium phosphate monobasic and sodium phosphate dibasic to create 25 mM concentrations in 18 MΩ nanopure water from an EMD Millipore system (Bedford, MA, USA). A 5 mM lipid concentration of sphingomyelin nanodiscs in background electrolyte (BGE) was used for all separations. Stock solutions (25 mM) of each atropisomer were prepared in acetone. Analytical samples were prepared from stock solutions by dilution to 62.5 μM of each atropisomer in BGE so that the injected sample contained only 0.5% acetone. EKC experiments were performed on an Agilent 3D CE

instrument with on-column DAD controlled by Agilent Chemstation software using 50 μm id fused-silica capillaries (Polymicro Technologies).

Capillaries with total length of 48.5 cm and effective length of 40 cm were flushed at the beginning of every day with 1.0 M NaOH. Between injections, the capillary was flushed with acetone, nanopure water, and buffered nanodisc solution. Analytes were injected with 35 mBar of pressure for 5 s and detected at 245 nm. All analyses were run at 15 kV applied voltage and 30°C using 5 mM sphingomyelin concentration of nanodiscs in BGE.

The migration time and the effective electrophoretic mobility of the nanodiscs in the BGE were estimated by using the method of Bushey and Jorgenson³⁷ using the negative water peak as the EOF marker and the migration times of six alkyl-phenyl ketone homologs: acetophenone, propiophenone, butyrophenone, valerophenone, hexanophenone, and heptanophenone. The Excel application Solver was used to determine the nanodisc migration time that gave the maximum r^2 for the plot of log retention factor versus homolog carbon number. Methylene selectivity, which is the average chromatographic selectivity between adjacent analogues, was calculated from the antilogarithm of the slope of the log k values versus the number of carbons in the alkyl chain of the homologous series of phenones³⁸.

The retention factors for all analytes were calculated using the following equation:

$$k = \frac{\mu_{eo} - \mu_{sol}}{\mu_{sol} - (\mu_{eo} + \mu_{PSP})}$$

Where μ_{eo} is the electroosmotic flow during the analyte run determined by the migration time of the water peak and μ_{sol} is the total (observed) electrophoretic mobility of the solute including the μ_{eo} . The μ_{PSP} is the electrophoretic mobility of the nanodiscs determined separately. Theoretical plate counts were calculated assuming Gaussian peaks and using the baseline peak widths as an estimate of four standard deviations.

The resolution between atropisomers was calculated using the following equation:

$$R_s = \frac{\Delta t_r}{w_{av}}$$

Where t_r is the separation between peaks in units of time, and w_{av} is the average width of the two peaks in units of time³⁹. The selectivity between atropisomers, α , is calculated as k_S/k_R .

The number of analyte molecules bound per nanodisc during separation was determined from the known analyte and lipid concentrations as well as the following equations:

$$F_{ND} = \frac{k}{k + 1}$$

Where k is the retention fraction and F_{ND} is the fraction of the analyte which is bound to the nanodiscs during the separation³⁹.

3. Results and Discussion

3.1 Nanodisc Characterization

The nanodiscs used for the analysis of atropisomer interactions with sphingomyelin are similar in composition to sphingomyelin nanodiscs analyzed in previously published work¹⁴. The nanodisc properties are reported in Table 1. The different ratios of copolymer to lipid used in synthesis 2.50:1.00 (w:w) vs 2.00:1:00 (w:w) led to small but statistically significant ($p=0.002$) differences in methylene selectivity, a measure of hydrophobicity.

The more hydrophobic environment with higher polymer ratio is contrary to previous results with 1,2-dimyristoyl-sn-glycero-3-phosphocholine lipid nanodiscs¹⁴. In the current experiments, the nanodisc with lower ratio of copolymer to lipid was used to decrease the background absorbance and allow detection of analytes at low concentrations.

3.2 Separation of (R)-(+)& (S)-(-)-1,1'-Bi-2-naphthol

Using 5 mM concentration of sphingomyelin nanodiscs in the BGE, atropisomers of 1,1'-Bi-2-naphthol were separated. The resolution parameters are listed in Table 2, average resolution was 2.41 ± 0.34 with an average theoretical plate value of $889 \times 10^3 \pm 429 \times 10^3$. As shown in Figure 2 the (R)-(+) stereochemistry of 1,1'-Bi-2-naphthol was less retained than the (S)-(-) stereochemistry.

The selectivity of 1.03 ± 0.00 represents consistent difference in affinity for the R and S configurations. The F_{ND} of 0.908 indicates that 90.8% of the analytes were bound to the nanodiscs during separation. Based on previous measurements of the size of similar nanodiscs⁴⁰ and the molecular area of sphingomyelin, $52.5 \text{ \AA}^2$,⁴¹ it can be approximated that there are between 156–192 lipids per nanodisc. Using this information and the F_{ND} of analyte bound to nanodisc it can be estimated that there are approximately 4 analytes bound per nanodisc during the separation.

In order to confirm that this separation was result of the chiral selectivity of sphingomyelin and not the result of an artifact each atropisomer was run as a single standard to determine that the compound was pure. The retention order of the atropisomers was determined by spiking the racemic mixture with one of the atropisomers and observing the changes in peak area. The racemic mixture was run in BGE without nanodiscs and in a solution of belt polymer in BGE with no separation observed. 1,2-dimyristoyl-sn-glycero-3-phosphocholine nanodiscs were synthesized using the same synthesis parameters as the sphingomyelin nanodiscs. When 1,2-dimyristoyl-sn-glycero-3-phosphocholine nanodiscs were utilized as a pseudostationary phase no atropisomer separation was observed. Combined, these results demonstrate that sphingomyelin, and not the copolymer belt, is responsible for the observed atropisomer selectivity.

Sphingomyelin contains two chiral centers in a 2S,3R configuration at the hydrophilic/hydrophobic interface, where previous analysis has shown that a majority of the hydrophilic/

hydrophobic partitioning occurs⁴². The separation of the (R)-(+)/(S)-(-) atropisomers could be a result of a cooperative interaction between the two chiral centers or the configuration produces a sterically selective interaction with the atropisomers in the same way that dipeptide polymerized surfactants have for the separation of 1,1'-Bi-2-naphthol in EKC⁴³.

After successful separation of the atropisomers a series of chiral compounds of varied chemistry and structure were analyzed to determine if sphingomyelin nanodiscs were more generally enantiomer or diastereomer selective. The compounds that were analyzed are listed in table 3. These compounds were chosen because they were of varying hydrophobicities with different levels of acidity and basicity. No resolution was observed for any of these enantiomers or diastereomers using sphingomyelin nanodiscs. These results confirmed previously published analysis by Mannock *et al.* that sphingomyelin did not have strong selectivity in interactions with enantiomers²⁸.

4. Conclusion

In conclusion, the chiral selectivity of sphingomyelin bilayers was studied using nanodisc EKC analysis. It was determined that (S)-(-)-1,1'-Bi-2-naphthol had measurably stronger affinity for the sphingomyelin nanodisc than that (R)-(+)-1,1'-Bi-2-naphthol, to the point where the two atropisomers were successfully resolved. Sphingomyelin bilayers are shown to have selective interactions with an atropisomer, but not enantiomers or diastereomers. This result also demonstrates the high sensitivity of nanodisc electrokinetic chromatography to small differences in affinity between bilayers and ligands, and that axial chirality might influence passive diffusion into sphingomyelin bilayers.

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Abbreviations

NEKC	Nanodisc Electrokinetic Chromatography
CE	Capillary Electrophoresis
EKC	Electrokinetic Chromatography
BGE	Background Electrolyte

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Highlights

- Nanodisc Electrokinetic Chromatography is used to study stereoselectivity in lipid bilayer membranes
- Sphingomyelin lipid bilayer membranes are shown to provide stereoselective interactions with the atropisomers (R)-(+)/(S)-(-)-1,1'-Bi-2-naphthol.
- Stereoselective interactions were not observed for a selection of enantiomers and diastereomers with varied structure.

Figure 1.
Atropisomer and Sphingomyelin

Figure 2.

Separation of (R)-(+)/(S)-(-)-1,1'-Bi-2-naphthol. Separation parameters: 5 mM sphingomyelin nanodiscs with 2.00:1.00 (w:w) Xiran 25010 copolymer belt to lipid ratio, in a 25 mM phosphate pH 7.0. Capillary dimensions: 48.5 cm $\times$ 50 μ m I.D. The injection was made with 35 mbar of pressure for 5 seconds. The operating voltage was 15 kV with detection at 245 nm. This separation averaged 102×10^4 theoretical plates.

Table 1.

Nanodisc electrokinetic properties. μ_{ep} is the electrophoretic mobility, α_{CH2} is the methylene selectivity, and N is the theoretical plate count from the phenone separation. All values reported +/- one standard deviation for n measurements.

Copolymer	Copolymer: Lipid Ratio (w: w)	Nanodisc Lipid Composition	°C	$\mu_{ep} 10^{-4} (cm^2/V*s)$	α_{CH2}	$N \times 10^3$	n
25010	2.00:1.00	Sphingomyelin	30	-4.17 ± 0.09	2.26 ± 0.06	214 ± 33	8
25010 ¹⁴	2.50:1.00	Sphingomyelin	30	-4.09 ± 0.03	2.37 ± 0.03	232 ± 24	7

Table 2.

1,1'-bi-2-naphthol Separation results. k is the retention factor, α the chromatographic selectivity, F_{ND} is fraction of the analyte bound to the nanodisc during the separation, N is the number of theoretical plates, and R_s the chromatographic resolution.

Atropisomer	k_R	k_S	$k_{average}$	α	F_{ND}	$N \times 10^3$	R_s	n
(R)-(+)/(S)-(-)1,1'-bi-2-naphthol	9.77 $\pm$ 0.79	10.07 $\pm$ 0.83	9.92 $\pm$ 0.81	1.03 $\pm$ 0.00	0.908 $\pm$ 0.007	889 $\pm$ 429	2.41 $\pm$ 0.34	3

Table 3.

Chiral analytes that showed no evidence of selectivity using sphingomyelin nanodiscs as a chiral selector.

Analytes	Form of stereochemistry
1-Phenyl-2-propyn-1-ol	Enantiomer
3-(α -Acetonyl-4-chlorobenzyl)-4-hydroxycoumarin	Enantiomer
Chlorthalidone	Enantiomer
Ephedrine	Diastereomer
Flavanone	Enantiomer
Furoin	Enantiomer
Homatropine Hydrobromide	Enantiomer
Hydrobezoin	Diastereomer
Ketoprofen	Enantiomer
Methyl DL mandelate	Enantiomer
Norphenylephrine Hydrochloride	Enantiomer
Omeprazole	Enantiomer
Pseudoephedrine	Diastereomer
Propranolol Hydrochloride	Enantiomer
Salbutamol	Enantiomer
Verapamil	Enantiomer