IMMOBILIZED ARTIFICIAL MEMBRANE (IAM) LIQUID CHROMATOGRAPHY AS A MODEL FOR ANTIMICROBIAL PEPTIDE PARTITIONING INTO CELL MEMBRANES: AN EVALUATION

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Abstract

Non-covalent immobilized artificial membrane reverse-phase high performance liquid chromatography was previously evaluated as a means whereby elution times for antimicrobial peptides from columns mimicking the lipid bilayers of different membrane systems might be used as a fast-screening method to compare relative binding effectiveness. Such a system would aid in the development of antimicrobial peptides that bind preferentially to model pathogenic systems and leave the host's membranes reasonably unaffected. A non-covalent approach allows for flexibility in membrane composition but was found to be inadequate for analysis of most peptides due to significant lipid loss at high acetonitrile concentrations. A covalent approach where phosphatidylcholine was amide-linked to the silica surface was examined to evaluate its use as a fast-screening method and compare its data to that collected from the non-covalent columns. Initial work with a 1-cm column proved ineffective due to problems with balancing flow rates with retention times, and work was shifted to a longer 10-cm column. Results suggested that peptides bind much more strongly to covalent columns than non-covalent ones, with the binding especially enhanced by the presence of cationic residues. These columns had lipid packing densities much lower than true membranes, indicating that the peptides were partitioning deep into the bonded phase of the columns rather than into the interfacial region of the phosphate head groups, as expected in situations of biologically-relevant lipid packing densities.

Introduction

The ability of antimicrobial peptides (AMPs) to partition into the phospholipid bilayers of cell membranes is a key factor in determining their overall biological activity (Brogden, 2005). In addition to displaying antimicrobial activity, these peptides display anticancer, antifungal, antimalarial (Apponyi, Pukala, Brinkworth, Masselli, Bowie, Tyler, Booker, et al., 2004), and even antiviral characteristics, including against the HIV virus (Lorin, Saidi, Belaid, Zairi, Baleux, Hocini, Belec, et al., 2005). Major public health concerns of today include the emergence of pathogenic bacteria having resistance to conventional antibiotics. As a result, there is significant research into novel alternatives to such treatments, one of them being antimicrobial peptides (Jiang, Vasil, Hale, Hancock, Vasil, & Hodges, 2008). These peptides are particularly attractive because they are usually part of the innate immune response of higher organisms and appear to act by degrading the barrier properties of cell membranes, leading to cytoplasmic leakage and ultimately death (Prenner, Kiricsi, Jelokhani-Niaraki, Lewis, Hodges, & McElhaney, 2005). The mode of action of AMPs via membrane disruption also poses a problem in their tendency to promote hemolysis (Jiang et al., 2008; Prenner et al., 2005). Hence, these peptides need to have their lipid specificity tuned in order to maximize their therapeutic value by increasing their antimicrobial activity and particularly by reducing their hemolytic activity. To gauge the effectiveness of AMPs, they are often introduced into a bacterial culture and the minimal inhibitory concentrations and LD_{50} values are determined (Prenner et al., 2005). However, an easy way of measuring the therapeutic index (preference for bacterial versus animal membranes) is required before these peptides are ready for animal trials and further drug development.

What is needed to study AMP activity is a fast-screening method that simulates *in vitro* the partitioning process of AMPs into the phospholipid bilayers of cell membranes (Fig. 1A). One option is to correlate reverse-phase high-performance liquid chromatography (RP-HPLC) retention times to AMP membrane activity. However, this technique represents only the hydrophobic component of the partitioning process. Other studies have shown that RP-HPLC relates poorly to biological assays such as drug-intestinal absorption, but the correlation is much improved with use of phospholipids as a bonded phase (Pidgeon, Ong, Liu, Qiu, Pidgeon, Dantzig, Munroe, et al., 1995). Phospholipids are amphipathic molecules, meaning that their interactions with amphipathic AMPs are also influenced by interactions with the lipid glycerol backbone and polar head groups, as well as with the non-polar hydrocarbon chains.

Non-covalent immobilized artificial membrane (NC-IAM) RP-HPLC was previously evaluated in our laboratory to determine whether elution of AMPs from columns mimicking different membrane systems could be used to compare relative binding effectiveness. Such a technique could aid in the development of AMPs that bind relatively strongly to systems resembling pathogens while leaving the host's cell membranes reasonably unaffected. These columns were prepared by passing solutions containing the lipid(s) of interest through the columns and allowing monolayers to form. The main advantage of NC-IAM RP-HPLC is that it is easy to change the lipid composition of these columns. By not covalently linking lipids, a single column may be used to gather retention times from numerous lipid systems. The absence of covalent linkages ensures that the lipids are free to laterally diffuse as they would in a real membrane. Results indicated that retention times increased with increasing membrane fluidity, but for peptides that elute with less than thirty percent acetonitrile (B), the times were always less than for a control column without a lipid monolayer. Further, the monolayers became unstable beyond forty percent B, resulting in significant lipid loss over sequential runs. This suggested that NC-IAMs were not suitable for analysis of either high molecular weight (>1000 Da) or highly lipophilic peptides. Also, for peptides that could be eluted beyond 40% B, retention times were slightly greater than those on the control column. A plausible explanation is discussed by Qiu and Pidgeon (1993). As Fig 1B shows, at high %B, the eluent becomes sufficiently non-polar and the head groups aggregate to exclude the solvent while maximizing the solvation of their non-polar chains. As a result, after a peptide partitions into the interfacial region, the head groups effectively shield the peptide from exposure to the eluent. A direct consequence of this is that the solvent system must become disproportionably non-polar in order to elute the peptide. This suggests that at high %B the elution time for these lipid columns may not accurately reflect relative retention behaviours on comparison to low %B-eluting peptides.

The limitations presented by NC-IAM RP-HPLC lead to an evaluation of a covalent approach where phosphatidylcholine (PC) was amide-linked to the silica surface (Fig. 1C). This column system was designed by Charles Pidgeon and commercialized by Regis Technologies of Morton Grove, IL, USA (Pidgeon et al., 1995). The inability for PC to lateral diffuse as in a real membrane and on a NC-IAM was proposed to not be a problem, as other researchers have demonstrated that most of the interaction with these ligands occurs at the head group-backbone boundary (Ong, Liu, Qiu, Bhat, & Pidgeon, 1995). Further, conformational freedom provided by the glycerol backbone, and in particular the head group region, makes the motional characteristics of the PC head groups the same whether or not the PC is immobilized (Qiu & Pidgeon, 1993). Hence, retention times for AMPs on the PC covalent column were studied with the expectation that retention times would mirror those obtained from the non-covalent PC column.

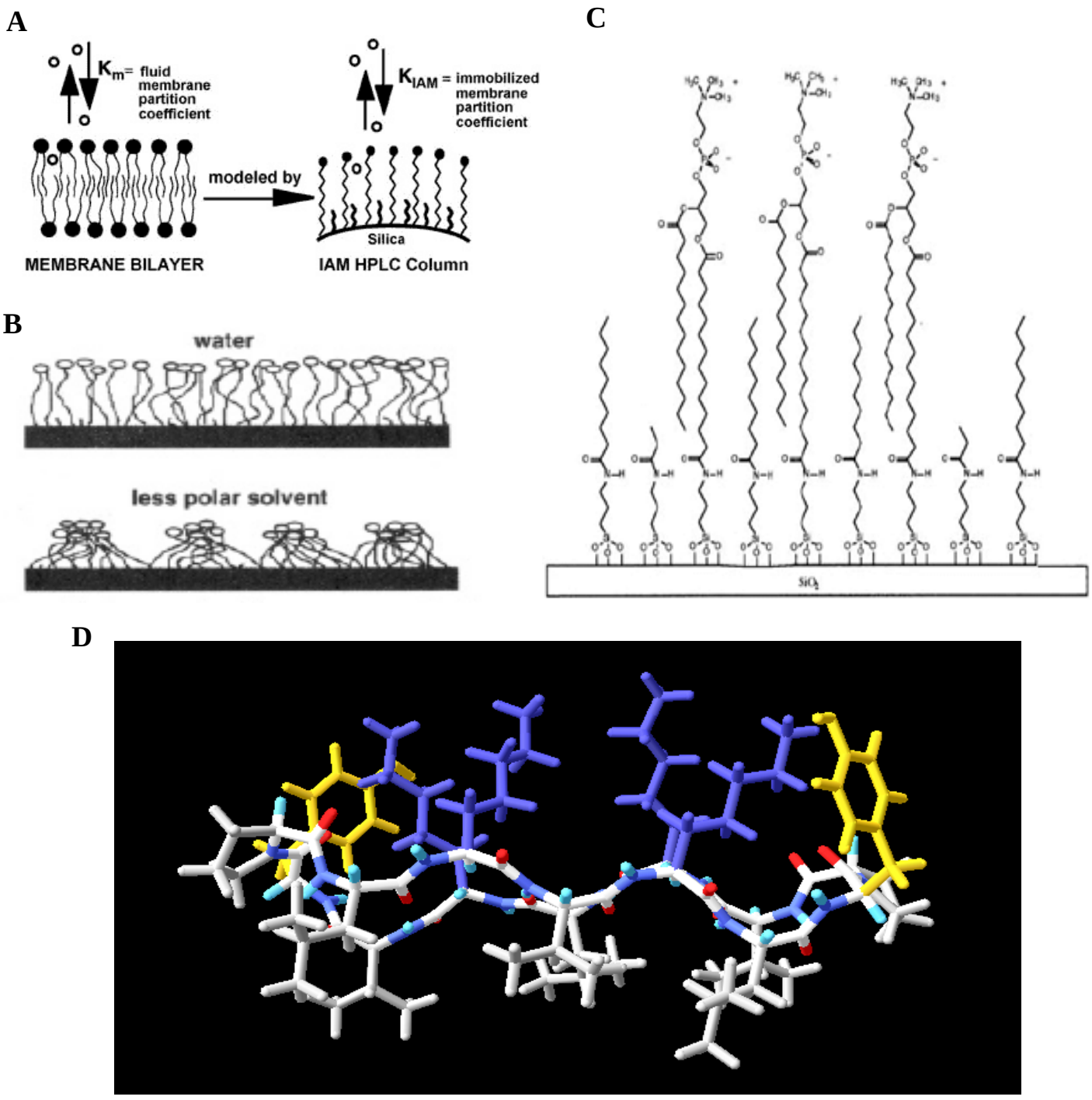

FIGURE 1: Overview of IAM Chromatography. A: The partitioning of a ligand, like a peptide, into a real biological membrane is mimicked by an IAM HPLC column. B: Effects of high concentrations of non-polar solvents (B) on artificial membranes. If a peptide has not eluted at low % B, the head groups aggregate to maximize the exposure of their non-polar chains to the solvent. As a result, after a peptide partitions into the interfacial region, the head groups shield the peptide, meaning that the solvent system must become disproportionably non-polar in order to elute the peptide. C: Structure of the IAM.PC.DD.2 column. Phosphatidylcholine is amide linked to the silica surface, and unreacted propyl-amine groups are first C10 end-capped and then C3 end-capped. D: Model of GS 14dk4. Positively charged (lysine) residues are in blue, polar residues in yellow, hydrophobic residues in white, and the main chain is coloured CPK. The peptide has a clear hydrophilic side and a hydrophobic side.

Experimental Procedures

The AMPs used in this investigation were synthesized by the Alberta Peptide Institute (University of Alberta, Edmonton, Canada) and Dr. R.S. Hodges (University of Colorado, CO, USA) by solid-phase techniques using Fmoc chemistry, and were shown to be >95% pure by HPLC and mass spectrometry. Peptides were dissolved in methanol at 1 mg/mL concentration. All solvents were of HPLC grade. In particular, four peptides were investigated: Gramicidin (GS) 4 [cyclo-(*d*YP*d*YP)], GS 6 [cyclo-(*d*YPK*d*YPK)], GS 14-dK4-V1A-L3A-V5A-L8A-V-10A-L12A (GS A) [cyclo-(AKA*d*KA*d*YPAKAKA*d*YP)], and GS 14dk4 [cyclo-(VKL*d*KV*d*YPLKVKL*d*YP)] (Fig.1D). The GS-14 family is a ring-expanded analog of Gramicidin S, [cyclo-(VOL*d*FPVOL*d*FP)], a cyclic 10 amino acid peptide derivative of gramicidin, produced by the Gram-positive bacteria *Bacillus Brevis*. As Fig. 1D shows, a common structural motif of these peptides is the presence of charged residues on one side of the peptide and hydrophobic residues on the other, with *d*-tyrosine and proline residues forming the corners.

An IAM Fast-Screen Mini Column Kit was obtained from Regis Technologies (Morton Grove, IL, USA) containing a 1 cm x 3 mm inner diameter (i.d.), 10 µm, 300Å IAM.PC.DD.2-packed column (Fig. 1C), as well as a 10 cm x 4.6 mm i.d. version of the same column. Columns were cleaned of lipophilic residue by eluting 100% acetonitrile at 1 mL/min and 25°C and then equilibrated in various pH 6.5 phosphate buffers (A) under the same conditions using a Hewlett Packard Agilent 1100 Series chromatograph (Waldbronn, Germany) and/or a Millipore Waters 600E System Control chromatograph with a Millipore Waters Lambda-Mac Model 401 LC Spectrophotometer (Milform, MA, USA). Peptides were injected on the column as 20 µL samples and eluted using either a 0-100% B (5 mM sodium phosphate pH 6.5 60%/40% acetonitrile/water) 0-36 min gradient; 0-100% B (30 mM ammonium phosphate pH 6.5 60%/40% acetonitrile/water) 0-36 min gradient; 0-100% B (10 mM sodium phosphate pH 6.5 50%/50% acetonitrile/water) 0-30 min gradient with 0%, 1%, 2%, or 5% v/v glycerol; or a 0-100% B (unbuffered acetonitrile) 0-60 min gradient. Due to its greater solubility, ammonium phosphate was used in place of sodium phosphate for preparing solvents with higher ionic strength. The elution was monitored by charting absorbance readings at 210 nm, and this data was analyzed and presented using the Origin software package (Microcal Software Inc., Northampton, MA, USA).

Results

Initial work with the 1-cm IAM.PC.DD2 mini-column proved ineffective due to problems balancing flow rates with retention times. Normal flow rates for these columns, according to the manufacturer's use guide, are 0.2-0.5 mL/min. However, the peptides studied would not elute in reasonable times at these rates. Elutions did occur when the flow rate was increased to 1 mL/min, but samples often appeared to elute as a non-reproducible series of aggregates or the signal from the peptide was lost in the fluctuations of the baseline due to uneven back pressure. As Fig. 2 shows, the signal for GS 14dk4, if present, is indistinguishable from the baseline. To rule out the possibility that the non-repeatable aggregate behaviour with multiple peaks appearing at greater than 40% acetonitrile concentrations might be due to interaction of the peptide with the silica base, the column was overloaded with Gramicidin S, one of the most hydrophobic peptides in our lab, eluting at 58% acetonitrile on the control column (data not shown), then equilibrated in phosphate buffer. Retention times though did not change (data not shown). Hence, the idea that reducing the column length from 15-cm (the length of the column used in previous NC-IAM studies) to 1-cm could be compensated by reducing the flow rate was shown to be invalid.

Further work proceeded with the 10-cm IAM.PC.DD.2 column and problems with the

uneven back pressure and noisy baselines were eliminated. However, for all peptides studied, regardless of secondary structure or amino acid composition, retention times were significantly increased compared to the NC-IAM columns with or without phospholipid (1-palmitoyl-2-oleoyl-phosphatidylcholine - POPC), as seen in Table 1. Peptides in the GS 14dk4 family would not elute in either unbuffered or 5-mM sodium phosphate solutions, unlike the NC-IAM columns. Fig. 3 shows chromatograms for GS A. GS A has the same structure as GS 14dk4 except that all of the hydrophobic amino acids have been replaced with alanine, making it the least hydrophobic peptide in the GS 14dk4 family. However, the peptide would not elute unless the eluent contained salt in the order of 30 mM concentration. Hence, the study of peptide interactions on this column became limited to GS 4, a hydrophobic tetrapeptide modeling the corners of GS 14dk4, and GS 6, a hexapeptide with two lysine residues inserted into the GS 4 sequence, modeling the cationic face of the GS 14dk4 family.

Fig. 4 compares retention times between the NC-IAM column and the covalent column for GS 4. Consistent with low %B eluting peptides, GS 4 elutes more quickly on the POPC NC-IAM relative to the same column without lipid, but is retained more strongly on the covalent column. The presence of 5 mM sodium phosphate in the eluent only slightly decreases the retention time compared to the unbuffered run, but this is starkly contrasted by GS 6 in Fig. 5. Unbuffered, GS 6 elutes at 78.5% acetonitrile, while in 5 mM sodium phosphate the retention is nearly halved, eluting at 43.1% acetonitrile. With two lysine residues, GS 6 is not as hydrophobic as GS 4, and this is confirmed by its elution at 18.6% acetonitrile on the non-lipid NC-IAM column compared to 19.7% for GS 4 (Table 1).

These initial results suggested that there were significant dissimilarities in the retention behaviour between the NC and covalent systems, prompting an investigation into the role ionic strength has on the binding properties of peptides on the covalent column. The effects of ionic strength on the retention of GS 4 (Fig. 6) were found to be relatively insignificant. Compared to the unbuffered run, higher ionic strength solutions increased elution times. This is the opposite result to when the same treatments were applied to GS 6 in Fig. 7. As ionic strength increased, retention time decreased, and the use of either sodium phosphate or ammonium phosphate led to the same effect. A 5 mM solution was sufficient to reduce the retention time by nearly half compared to the unbuffered run, and increasing ionic concentrations marginally enhanced this behaviour. These results were consistent with GS 6 interacting electrostatically as well as hydrophobically with the column's bonded phase, indicating that the two lysine residues determined a large component of the partitioning nature of the peptide.

Further, investigations into hydrogen-bonding effects on the retention behaviours of both GS 4 and GS 6 on the covalent column showed similar results (Fig. 8 and 9). Glycerol was added to the eluents as 1%, 2%, and 5% v/v concentrations at constant ionic strength (10 mM sodium phosphate). Higher concentrations were not studied because beyond 5%, the eluents became too viscous to pump and presented problems with clogging the chromatograph. For GS 4, the 1% solution had no effect, whereas a small decrease in retention time was seen for the 2% and 5% solutions, with the 5% retention time being marginally less than the 2%. For GS 6, all mixtures slightly decreased retention times with increasing glycerol concentrations, with the greatest decrease occurring between the 2% and 5% solutions. Overall, the decrease in retention times was not as significant as the effect seen with increasing ionic concentration.

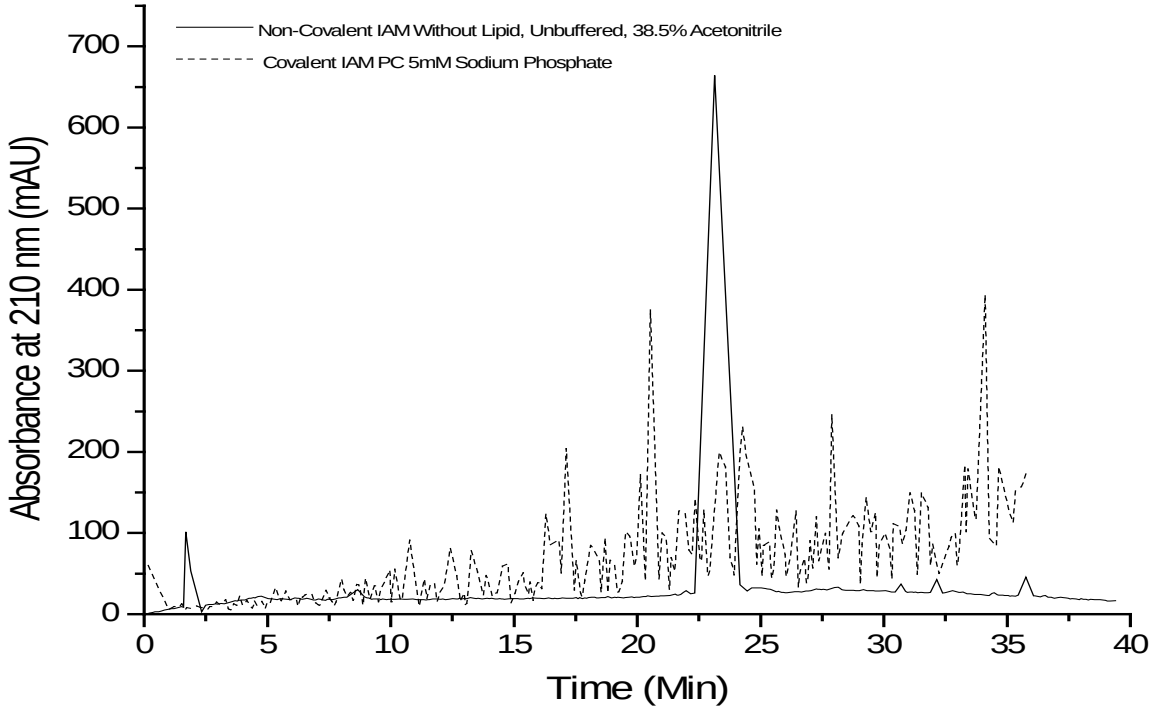

FIGURE 2: Comparison of non-covalent 15-cm column (without lipid) to 1-cm IAM.PC.DD.2 mini-column for GS 14dk4. Samples run on a 0-100% acetonitrile 0-60 min linear gradient at 25°C and 1 mL/min flow rate. Buffered runs carried out at pH 6.5. The chromatogram for the mini-column has an extremely noisy baseline which would mask any signal from the peptide. A flow rate of at least 1 mL/min was required on the mini-column in order to see any peptide elution, however, this flow rate led to uneven back pressure resulting in problems obtaining a stable baseline, particularly at higher acetonitrile concentrations.

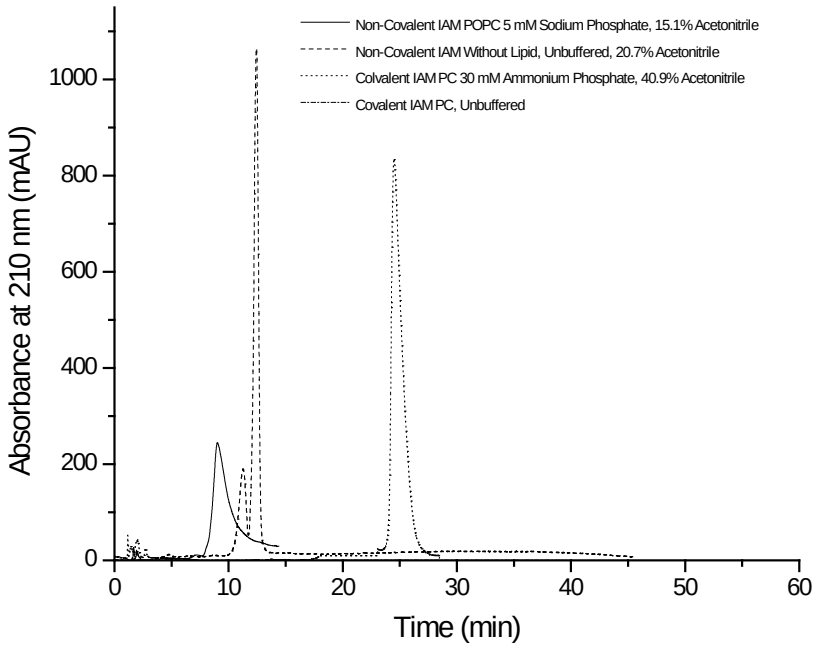

FIGURE 3: Comparison of retention times between non-covalent and covalent IAM columns for GS A. All samples run on a 0-100% acetonitrile 0-60 min linear gradient at 25°C and 1 mL/min flow rate. Buffered runs carried out at pH 6.5. The decrease in retention time for GS A for the non-covalent-IAM compared to the same column without lipid is consistent with the peptide partitioning into the lipid backbone interface rather than partitioning deeper into the column's bonded phase. For the covalent column, eulents needed to contain upwards of 30 mM of salt before elution would take place, suggesting that electrostatic interactions are very prevalent in GS A's interaction with the covalent column's bonded phase. The presence of salt competes with the head groups for interaction with the peptide, significantly decreasing the retention time.

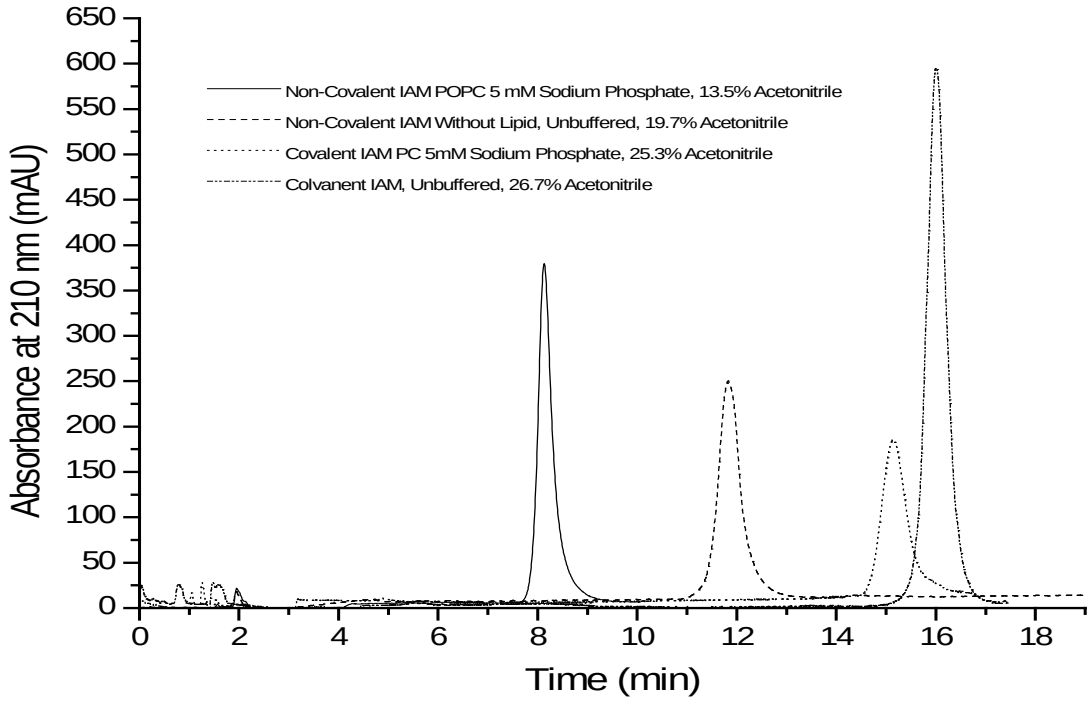

FIGURE 4: Comparison of retention times between non-covalent and covalent IAM columns for GS 4. All samples run on a 0-100% acetonitrile 0-60 min linear gradient at 25°C and 1 m L/min flow rate. Buffered runs carried out at pH 6.5. The decrease in retention time for GS4 for the non-covalent-IAM compared to the same column without lipid is consistent with the peptide partitioning into the lipid backbone interface rather than partitioning deeper into the column's bonded phase. For the covalent IAM, the presence of salt decreases the retention only slightly, suggesting that this overall hydrophobic peptide has little interaction with the phosphate head groups.

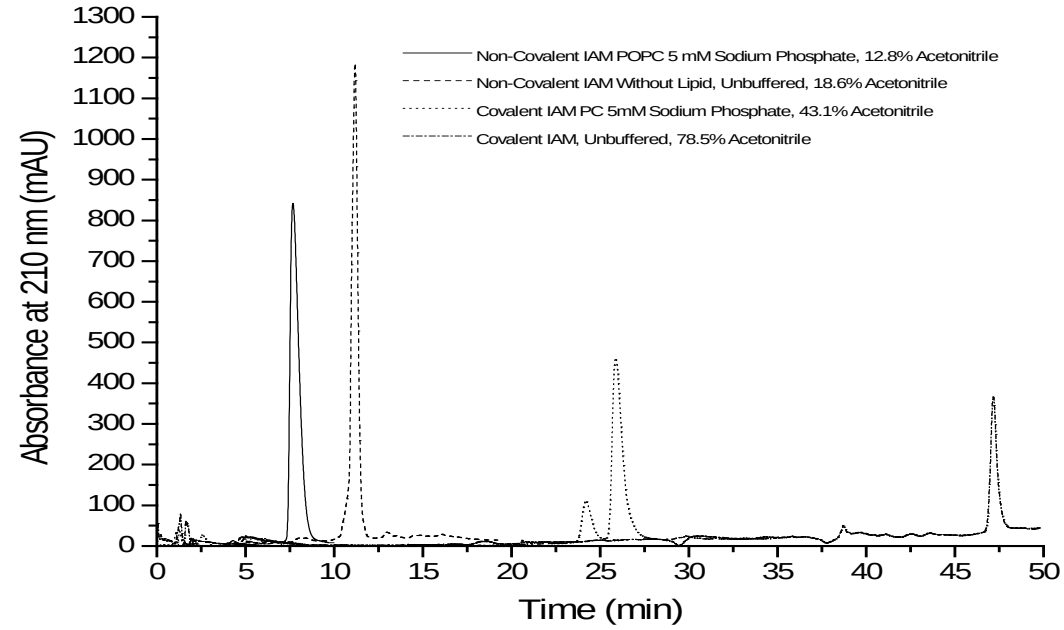

FIGURE 5: Comparison of retention times between non-covalent and covalent IAM columns for GS 6. All samples run on a 0-100% acetonitrile 0-60 min linear gradient at 25°C and 1 mL/min flow rate. Buffered runs carried out at pH 6.5. The decrease in retention time for GS 6 for the non-covalent-IAM compared to the same column without lipid is consistent with the peptide partitioning into the lipid backbone interface rather than partitioning deeper into the column's bonded phase. The retention times for both runs on this column are less than that for GS 4, since the addition of two lysine residues decreases the overall hydrophobicity of the peptide. For the covalent IAM, retention times for both the buffered and unbuffered runs are increased and dramatically different, suggesting partitioning deep into the bonded phase and interaction of the lysine residues with the negative charge of the phosphate head groups. The presence of salt competes with the head groups for interaction with the peptide, significantly decreasing the retention time.

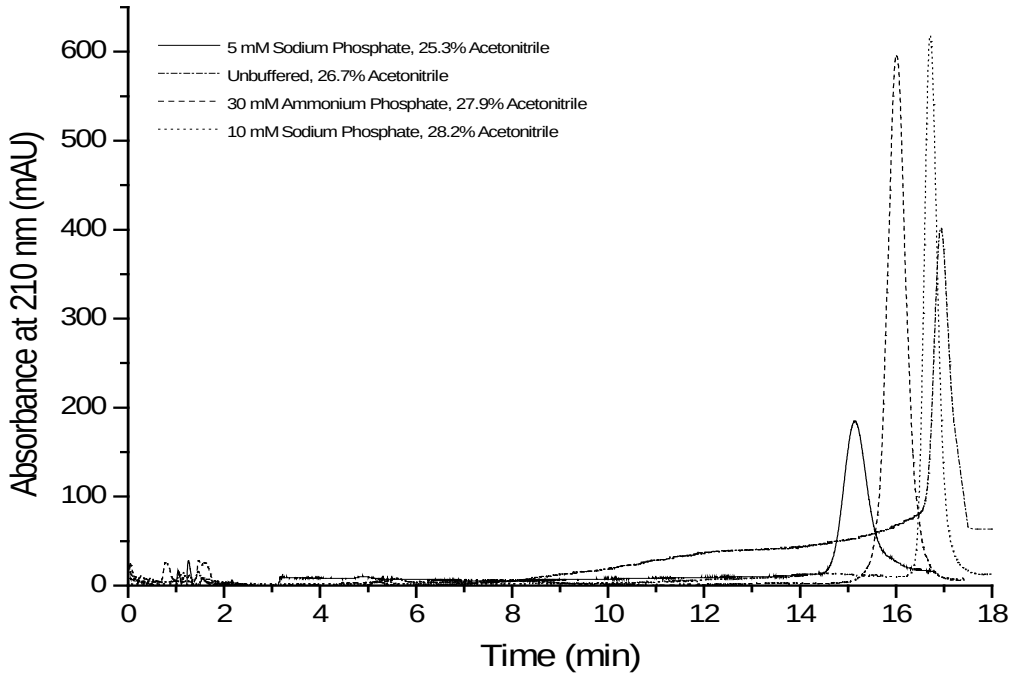

FIGURE 6: Comparison of retention times for the IAM.PC.DD.2 covalent column with changing ionic strength for GS 4. All samples run on a 0-100% acetonitrile 0-60 min linear gradient at 25°C and 1 mL/min flow rate. Buffered runs carried out at pH 6.5. Overall, increasing the ionic strength slightly increases the retention time, consistent with the eluent becoming more polar and GS 4 being an overall hydrophobic peptide. Ionic interactions do not appear to play a significant role in the partitioning of GS 4 into the membrane.

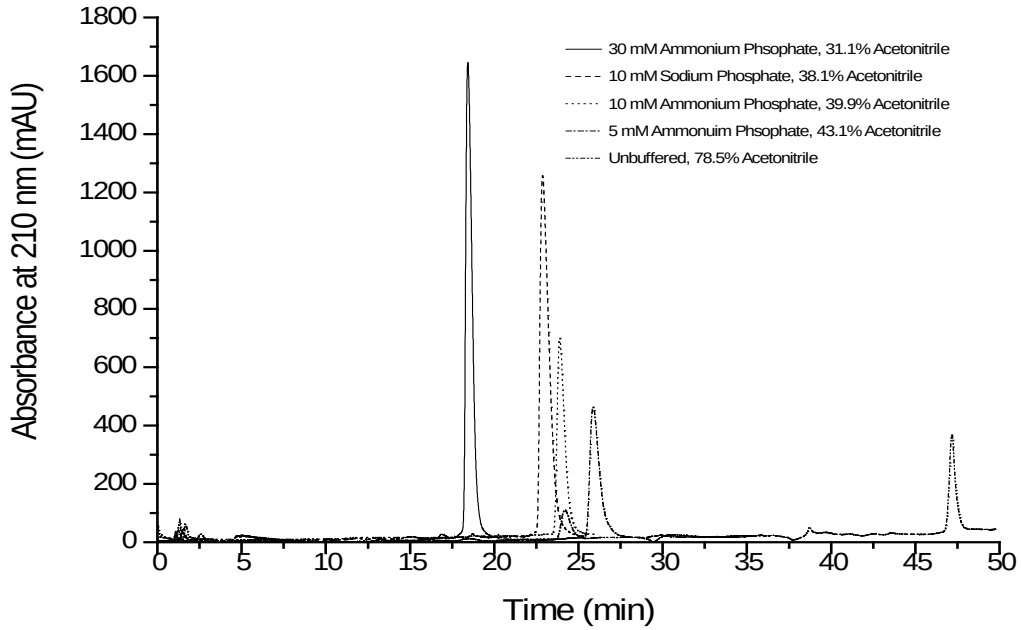

FIGURE 7: Comparison of retention times for the IAM.PC.DD.2 covalent column with changing ionic strength for GS 6. All samples run on a 0-100% acetonitrile 0-60 min linear gradient at 25°C and 1 mL/min flow rate. Buffered runs carried out at pH 6.5. As ionic strength is increased, the retention time for GS 6 decreases, indicating that the presence of salt competes with the negative charge of the phosphate head group for interaction with the peptide's charged (lysine) side chains. Ammonium phosphate is more soluble than sodium phosphate and was used for the 30 mM run since the upper limit of solubility for sodium phosphate in the eluent used was approximately 10 mM. There is little difference in retention time between the 10 mM ammonium phosphate and 10 mM sodium phosphate runs. More significant is the drastic decrease in retention time by nearly half upon addition of 5 mM of sodium phosphate when compared to the unbuffered run.

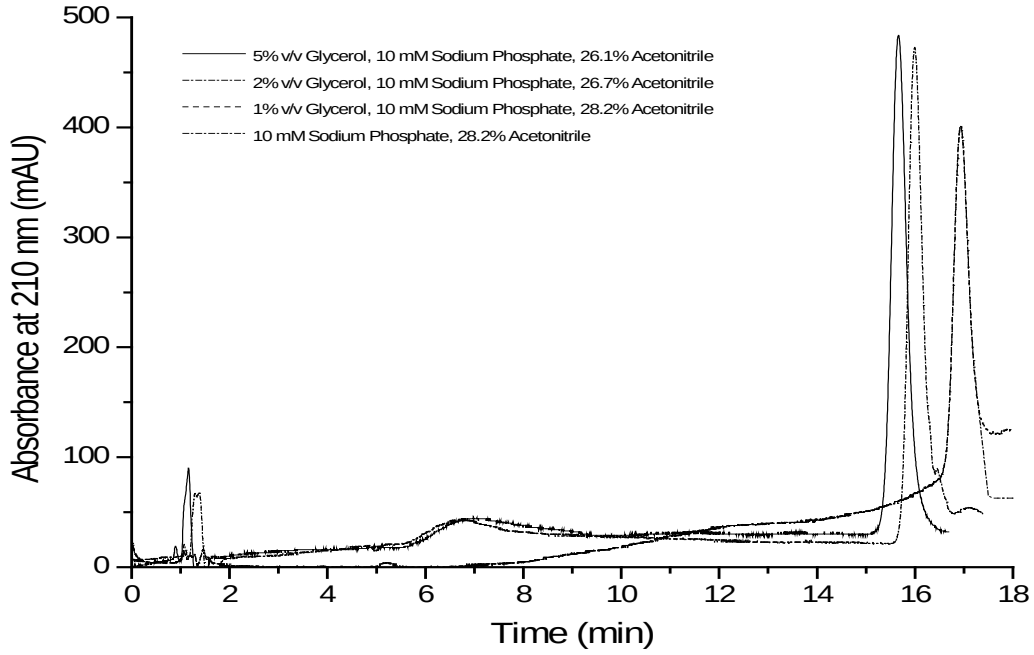

FIGURE 8: Comparison of retention times for the IAM.PC.DD.2 covalent column with changing glycerol concentrations for GS 4. All samples run on a 0-100% acetonitrile 0-60 min linear gradient at 25°C, pH 6.5, and 1 mL/min flow rate. Glycerol is a potent hydrogen-bond donor and was added to the solvent system to compete with the peptide for hydrogen-bond acceptor sites on the phosphate head groups. No effect was observed at 1% v/v glycerol, while very slight decreases in retention time were seen with the 2% and 5% v/v glycerol solutions, suggesting that hydrogen bonding plays only a small role in the peptides interaction with the column's bonded phase.

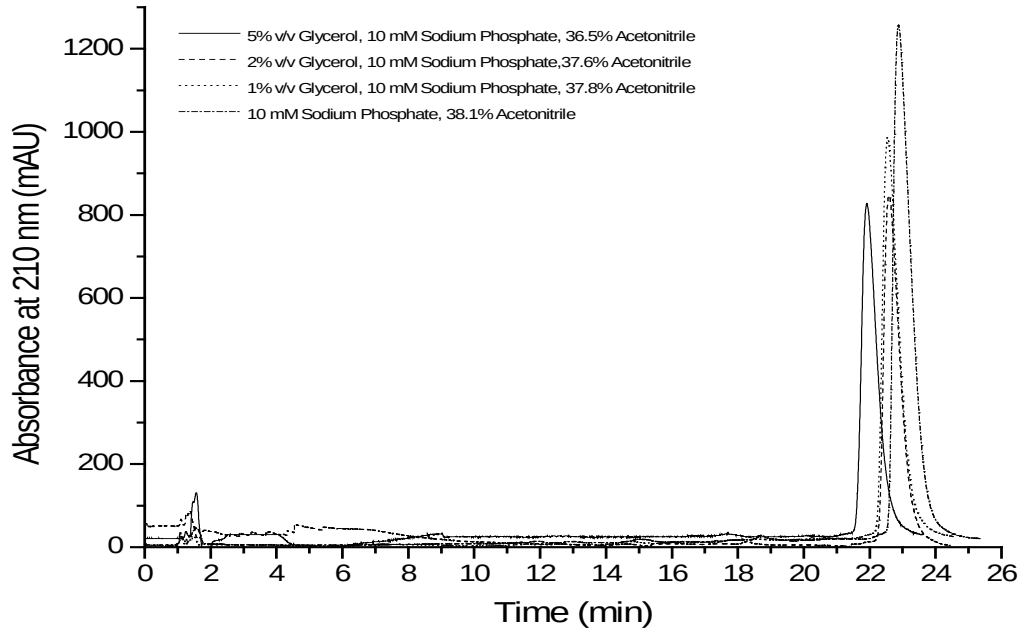

FIGURE 9: Comparison of retention times for the IAM.PC.DD.2 covalent column with changing glycerol concentrations for GS 6. All samples run on a 0-100% acetonitrile 0-60 min linear gradient at 25°C, pH 6.5, and 1 mL/min flow rate. Glycerol is a potent hydrogen-bond donor and was added to the solvent system to compete with the peptide for hydrogen-bond acceptor sites on the phosphate head groups. Very slight decreases in retention times occurred with increasing glycerol concentrations, suggesting hydrogen bonding has a limited role in the peptides interaction with the column's bonded phase compared to ionic and hydrophobic effects.

Peptide	Column	"A" Solvent System	"B" Solvent System	Linear AB Gradient	Elution % CH_3CN
GS A	non-covalent POPC	5 mM sodium phosphate H_2O	5 mM sodium phosphate 60/40 H_2O / CH_3CN	0-100% B 36-min	15.1
GS A	non-covalent no lipid	Unbuffered H_2O	Unbuffered CH3CN	0-100% B 60-min	20.7
GS A	IAM.PC.DD.2	Unbuffered H_2O	Unbuffered CH3CN	0-100% B 60-min	no elution
GS A	IAM.PC.DD.2	30 mM ammonium phosphate H_2O	30 mM ammonium phosphate 60/40 H_2O / CH_3CN	0-100% B 36-min	40.9
GS 4	non-covalent POPC	5 mM sodium phosphate H_2O	5 mM sodium phosphate 60/40 H_2O / CH_3CN	0-100% B 36-min	13.5
GS 4	non-covalent no lipid	Unbuffered H_2O	Unbuffered CH_3CN	0-100% B 60-min	19.7
GS 4	IAM.PC.DD.2	Unbuffered H_2O	Unbuffered CH_3CN	0-100% B 60-min	26.7
GS 4	IAM.PC.DD.2	5 mM sodium phosphate H_2O	5 mM sodium phosphate 60/40 H_2O / CH_3CN	0-100% B 36-min	25.3
GS 4	IAM.PC.DD.2	10 mM sodium phosphate H_2O	10 mM sodium phosphate 50/50 H_2O / CH_3CN	0-100% B 30-min	28.2
GS 4	IAM.PC.DD.2	30 mM ammonium phosphate H_2O	30 mM ammonium phosphate 60/40 H_2O / CH_3CN	0-100% B 36-min	27.9
GS 4	IAM.PC.DD.2	10 mM sodium phosphate H_2O, 1% v/v glycerol	10 mM sodium phosphate 50/50 H_2O / CH_3CN, 1% v/v glycerol	0-100% B 30-min	28.2
GS 4	IAM.PC.DD.2	10 mM sodium phosphate H_2O, 2% v/v glycerol	10 mM sodium phosphate 50/50 H_2O / CH_3CN, 2% v/v glycerol	0-100% B 30-min	26.7
GS 4	IAM.PC.DD.2	10 mM sodium phosphate H_2O, 5% v/v glycerol	10 mM sodium phosphate 50/50 H_2O / CH_3CN, 5% v/v glycerol	0-100% B 30-min	26.1
GS 6	non-covalent POPC	5 mM sodium phosphate H_2O	5 mM sodium phosphate 60/40 H_2O / CH_3CN	0-100% B 36-min	12.8
GS 6	non-covalent no lipid	Unbuffered H_2O	Unbuffered CH_3CN	0-100% B 60-min	18.6
GS 6	IAM.PC.DD.2	Unbuffered H_2O	Unbuffered CH_3CN	0-100% B 60-min	78.5
GS 6	IAM.PC.DD.2	5 mM sodium phosphate H_2O	5 mM sodium phosphate 60/40 H_2O / CH_3CN	0-100% B 36-min	43.1
GS 6	IAM.PC.DD.2	10 mM sodium phosphate H_2O	10 mM sodium phosphate 50/50 H_2O / CH_3CN	0-100% B 30-min	38.1
GS 6	IAM.PC.DD.2	10 mM ammonium phosphate H_2O	10 mM ammonium phosphate 50/50 H_2O / CH_3CN	0-100% B 30-min	39.9
GS 6	IAM.PC.DD.2	30 mM ammonium phosphate H_2O	30 mM ammonium phosphate 60/40 H_2O / CH_3CN	0-100% B 36-min	31.1
GS 6	IAM.PC.DD.2	10 mM sodium phosphate H_2O, 1% v/v glycerol	10 mM sodium phosphate 50/50 H_2O / CH_3CN, 1% v/v glycerol	0-100% B 30-min	37.8
GS 6	IAM.PC.DD.2	10 mM sodium phosphate H_2O, 2% v/v glycerol	10 mM sodium phosphate 50/50 H_2O / CH_3CN, 2% v/v glycerol	0-100% B 30-min	37.6
GS 6	IAM.PC.DD.2	10 mM sodium phosphate H_2O, 5% v/v glycerol	10 mM sodium phosphate 50/50 H_2O / CH_3CN, 5% v/v glycerol	0-100% B 30-min	36.5

TABLE 1: Summary of solvent system conditions for peptide elutions. Non-covalent column was a Vydac 218TP5415 15 cm x 4.6 mm I.D. 5 µm-particle C_{18} column, and the IAM.PC.DD.2 column was a 10 cm x 4.6 mm I.D. 10 µm-particle column. All solutions containing phosphates were buffered to pH 6.5, with elutions carried out at 1 mL/min and 25°C. The rate of change in effective CH_3CN concentrations in all runs was 1.67%/min, with elution % CH_3CN being the % CH_3CN of the system at the moment of peptide elution.

Discussion

From the covalent IAM retention times gathered on the IAM.PC.DD.2 column, the peptides studied bind much more strongly to the covalent columns than both the non-covalent and non-lipid control columns, with the binding especially enhanced by the presence of cationic residues. This suggests that there is a distinct difference in the partitioning behaviours of AMPs into the covalently bonded phase relative to the non-covalent system.

It was postulated that the 1-cm mini-column could be used as an economical means of comparing the retention behaviours of covalent columns to the NC-IAM data previously obtained. Even though the NC-IAM column was 15 cm long, it was assumed that the shorter column length could be compensated for by reducing the flow rate. Nevertheless, at low flow rates, the elution times were significantly increased. At 1mL/min, the retention times did decrease, however they were not consistent from run to run. Further, the uneven back pressure at this rate may explain the apparent aggregate elution and/or noisy baseline. Even after the column was overloaded with the very hydrophobic Gramicidin S peptide, the lack in change of retention time ruled out silica interactions. Further, if silica interactions were a significant contributor to the increase in retention times, the effect should have been decreased with increased peptide size, as it would be more difficult for a large peptide compared to a small peptide to partition through the bonded phase to the silica base. However, the increase in retention times was consistent for all peptides studied, suggesting that these columns are not suitable for peptide elution requiring flow rates of at least 1 mL/min.

It is for this reason that the evaluation was continued with a 10 cm x 4.6 mm version of the mini-column. Problems with irregular back pressure at 1 mL/min were eliminated, and as a result, the multiple-peak behaviour seen in the 1-cm column disappeared. However, retention times were still much greater than non-lipid control and NC-IAM columns, presenting a paradox. Given that the particle size of the IAM.PC.DD2 was 10 µm, larger than the 5 µm particle of the NC-IAM column, and the overall hydrophobicity was reduced by the presence of zwitterionic head groups, the retention times from the IAM.PC.DD2 column were anticipated to mirror those obtained from the NC-IAM column when it was loaded with POPC. Such times would also be less than those for peptides eluted from the NC-IAM column without the phospholipid monolayer. This agreement was seen by Luo, Zheng, and Cheng (2007) on comparison of retention times for pyrimidine derivatives. However, analytes with highly polar substituents capable of significant hydrogen bonding or electrostatic interactions were retained longer on the IAM column, leading the authors to conclude that the retention behaviour of such compounds on biomembrane-mimic IAM phases were complex.

The difference in retention behaviors between the covalent and non-covalent systems may be due to different lipid packing densities. Ideally, an *in vitro* model of a biological membrane should have a packing density analogous to a real membrane in order to draw valid conclusions from ligand-lipid partitioning experiments. The non-covalent column was shown by Krause, Dathe, Wieprecht, and Bienert (1999) to have a density of 85$Å^2$ per molecule of phospholipid, reasonably close to the value of 60-70 $Å^2$ in phospholipid bilayers. This corresponds to a ratio of 158 µmol PC per gram of stationary phase. According to Ong and Pidgeon (1995), the developers of the covalent IAM technology, the IAM.PC.DD.2 column (referred to as $^{ester}IAM.PC^{C10/C3}$) contained 98 µmol PC per gram of stationary phase. However, contact with Regis Technologies indicated that the PC concentration of the column used is this experiment was 16 µmol PC per gram of stationary phase, or ten percent of the density in the Krause columns. This would suggest that these covalent columns, as commercialized by Regis Technologies, are not appropriate for

comparing the partitioning behaviours between the two models, given the enormous difference in lipid packing between the two systems.

A densely-packed membrane is necessary in order to establish compartmentalization of ions, proteins, and other cell components, and prevent non-specific transport across the bilayer. More specific to the context of this project, a lower immobilized PC density results in a lower interfacial barrier to solute transport into the hydrocarbon region of the bonded phase (Ong & Pidgeon, 1995). With a tight packing, the phosphate head groups effectively limit peptide partitioning into only the interfacial region. Further, the phosphates themselves shield their own charges from ligands that partition into such membranes. Hence, in a biological membrane, electrostatic and hydrophobic interactions with a peptide are reduced largely by the increased resistance to penetration.

With a reduced lipid packing density, peptides can penetrate beyond the interfacial head groups and embed within the bonded phase. The higher hydrocarbon surface area available for contact with the peptide can increase the van der Waals interactions between the hydrophobic components of the peptides and the bonded phase, amplifying the affinity of the peptide to the column (11). It is largely for this reason that all peptides studied were retained much longer on the IAM.PC.DD.2 column than the NC-IAM columns. Electrostatic effects appeared to be much more crucial in describing the partitioning than van der Waals forces when cationic residues were present within the peptide, as particularly seen in the inability to elute GS A with unbuffered eluents, and the large increase in acetonitrile concentration required for elution of GS 6 in unbuffered conditions relative to buffered runs. If the partitioning was only into the interfacial region as predicted in a normal membrane, the cationic charge of the choline portion of the head groups should have attenuated the electrostatic interaction and minimized the vast difference in retention times seen for cationic residues.

The difference between the GS 4 and GS 6 peptides was related to the presence of two lysine residues in GS 6, which reduced the overall hydrophobicity of the peptide relative to GS 4. Hence, GS 6 should have eluted more quickly than GS 4 under identical conditions, and this conclusion was observed for the NC-IAM columns, but not on the IAM.PC.DD2 column. The reduction in retention time by almost half for GS 6 in the 5 mM sodium phosphate run compared to the unbuffered run suggested that the lysine residues of GS 6 interacted electrostatically with the negative charge of the phosphate head groups of the zwitterionic PC moiety. Addition of salt to the eluent introduced electrostatic competition for the phosphate head groups, meaning that the ionic component of the partitioning of GS 6 was decreased. This is plausible because GS 4, a purely hydrophobic peptide, showed little change in retention times under the same circumstances. The overall increased retention times for both GS 4 and 6 on the IAM.PC.DD2 column relative to the NC-IAM columns indicated that hydrophobic interactions played a more significant role in the partitioning process on the covalently bonded phase.

Taillardat-Bertschinger, Marca Martinet, Carrupt, Reist, Caron, Fruttero, and Testa (2002) also argued that there is dissimilar partitioning of hydrophilic solutes into the IAM.PC.DD.2 bonded phase and PC liposomes. In their study, the interactions of β-blockers with the two systems were examined, and it was concluded that electrostatic effects played only a minor role in IAM retentions. Their justification was based on the smaller density of phospholipids in IAMs compared to liposomes, as well as the lack of lateral and axial mobility in IAM columns and their monolayer nature. This is in contrast to the conclusions from this investigation with peptides. However, the authors did suggest that hydrogen bonding played only a minor role in IAM retentions, which is in agreement with the glycerol runs for GS 4 and 6. However, it may be that the hydrophobic and electrostatic interactions observed in the IAM.PC.DD.2

system overwhelmed the nature of the peptide partitioning, and that hydrogen bonding may play a much more significant role when the ability for electrostatic interactions is muted with higher lipid densities in normal membranes.

The key difference between this study and those conducted by others like Luo *et al.* (2007) and Taillardat-Bertschinger *et al.* (2002) on the IAM.PC.DD2 system rests in the size of the solutes studied. Virtually all published research for these models makes use of small drugs such as pyrimidine derivatives that are often smaller than 300 Da. However, in our circumstance, solutes being examined contain 14 to 26 amino acids with molecular weights spanning 1700 to 3000 Da. Even though retention data correlated well for these drugs to intestinal absorption studies while the same is not true for retention data on ODS (octodecyl) (non-lipid NC-IAM) columns (Pidgeon et al., 1995), the question arises whether there is a critical lipid density required to perform a valid *in vitro* experiment that is dependent on the parameters of the solute of interest, such as size, overall hydrophobicity, and charge. If so, then the possibility exists that a lower packing density is sufficient for the study of small analytes. Hence, because the majority of research has been focused on this area, inadequacies with the IAM.PC.DD2 system and similar models may only begin to be become apparent when the range of solutes examined starts to include very large and/or charged ligands like AMPs.

Another fundamental difference between this evaluation and other on small solutes rests in the nature of the eluents used. For small solutes with limited van der Waals surfaces and/or low charge densities, eluents are typically aqueous sodium phosphate buffers (8) or contain low proportion of organic solvent, such as 20:80 (v/v%) methanol/sodium phosphate buffers (9). At low organic solvent concentrations, the eluent still behaves much like water and is only capable of solvating the interfacial region on the IAM. As described earlier and illustrated in Fig. 1B, as the eluent becomes increasingly non-polar, such as upon the addition of a large volume percentage of acetonitrile, the polar head groups begin to cluster together to exclude the eluent while the non-polar lipid chains become solvated (Qiu & Pidgeon, 1993). Elution of most peptides examined in this study required acetonitrile concentrations well beyond 40%. Therefore, while Taillardat-Bertschinger *et al.* argue that electrostatic interactions are not very important in the IAM.PC.DD.2 system due to low lipid packing, the head group aggregation effect can increase the overall charge density available for interaction with individual solute molecules as acetonitrile concentration increases. Hence, the entrapment of solute deep within the bonded phase by aggregated head groups makes understanding the elution process of cationic peptides on the IAM.PC.DD.2 column even more intricate.

Overall, determining the complex retention behavior of AMPs based on size, charge, and hydrophobicity requires an *in vitro* model that mimics a real biological membrane as closely as possible in order to minimize extraneous effects. Questions like what are the dominating forces behind peptide/lipid interactions will remain improperly answered until columns packed with a lipid density matching true membranes, or other systems providing accurate fast-screening models that are stable under high organic solvent concentrations, are developed.

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