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Antimicrobial properties of a lipid interactive α-helical peptide VP1 against *Staphylococcus aureus* bacteria

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Abbreviations

<μH>, amphiphilicity; DMPE, 1,2-Dimyristoyl-*sn*glycero-3-phosphoethanolamine; DMPG, 1,2-Dimyristoyl-*sn*-glycero-3-phospho-*sn*-1-glycerol; FTIR, Fourier transform infra red; <H>, hydrophobicity; HEPES, N-[2-Hydroxyethylpiperazine-N'-[2-ethanesulphonic acid]; Tris, Tris{hydroxymethyl} aminomethane.

Key words VP1, α-helical, lipid monolayer, antimicrobial

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ABSTRACT

Theoretical analysis indicates peptide VP1 forms a membrane interactive amphiphilic α-helix with antibacterial properties. Fourier transform infra-red based analyses showed VP1 to be α-helical (45%) in the presence of vesicle mimics of membranes from *S. aureus* and to induce increases in the fluidity of these vesicles, as indicated by a rise in wavenumber of *circa* 0.5 to 1.0 cm^{-1}. The peptide induced surface pressure increases of 5 mN m^{-1} in monolayer mimics of *S. aureus* membranes confirm the formation of an membrane interactive α helix. These interactions appeared to involve significant hydrophobic and electrostatic contributions as VP1 induced comparable surface pressure changes in anionic (5.5 mN m^{-1}) and zwitterionic (4 mN m^{-1}) lipid monolayers. It is suggested that whilst efficacy requires further sequence specific information, the peptides generic structure which provides the basis for its broad antimicrobial activity.

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1. Introduction

AMPs produced by the innate immune system are strong potential candidates in the ongoing search for new, therapeutically useful antibiotics with novel mechanisms of action [1-7]. Key features in this potential activity are the diversity of microbes efficiently killed by these peptides and the fact that, in contrast to conventional antibiotics, they do not normally appear to induce resistance in target organisms. It is generally accepted that the susceptibility of microbes to AMPs can be largely ascribed to the relatively non-specific nature of the antimicrobial mechanisms used by these peptides. All currently known AMPs are lipid interactive, either passing through the membrane to attack intracellular targets or, as in most cases, using direct invasion of the microbial membrane itself as their primary killing mechanism [8-12]. In general, studies to elucidate the mechanisms underlying these processes of membrane interaction have focused on AMPs that adopt an α-helical structure (α-AMPs) and have led to the Shai-Huang-Matsazuki (SHM) model, which hitherto, appears to best describe the antimicrobial action of these peptides. Essentially, the SHM model proposes that "carpeting" of the microbial membrane with α-AMPs leads to the displacement of membrane lipid, alterations to membrane structure and either microbial membrane destruction or peptide internalization [13]. However, more recently, theoretical analyses have suggested that the use of oblique orientated α-helical architecture, or tilted peptides, may feature in the microbial killing mechanisms of many α-AMPs [14]. This form of secondary structure, possesses an asymmetric distribution of hydrophobicity along the α-helical long axis, which causes a protein α-helix to penetrate membranes at a shallow angle, thereby disturbing membrane lipid organisation and compromising bilayer integrity [15]. The predictions of Dennison *et al.*, [14] regarding the importance of oblique structures for

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antimicrobial function have recently been supported by studies on naturally occurring α-AMPs [16].

Previous studies have identified VP1 within the lipid interactive smaller subunit of m-calpain [17] as having the potential to form a lipid interactive α-helical segment [18] that has characteristics similar to known antimicrobial peptides (AMPs) [14]. The potential of VP1 to act against Gram-positive organisms based solely on its general structural properties was therefore investigated.

2. Materials and Methods

2.1 Materials

VP1 (GTAMRILGGVI) was supplied by Severnbiotech (UK), produced by solid state synthesis and purified by HPLC to greater than 95%, which was confirmed by MALDI mass spectrometry. Nutrient broth was purchased from Amersham Bioscience (UK). Buffers and solutions for monolayer experiments were prepared from Milli-Q water. N-[2-Hydroxyethylpiperazine-N'-[2-ethanesulphonic acid] (HEPES), Tris{hydroxymethyl} aminomethane (Tris) and all other reagents were purchased from Sigma (UK).

2.2 Theoretical analysis of VP1

VP1 was graphically represented as an idealised α-helix using Winpep software [19] assuming an amino acid residue side chain angular periodicity of 100°. Using a moving window of 11 residues, the mean hydrophobic moment, $\langle \mu H \rangle$, and the corresponding mean hydrophobicity, $\langle H_0 \rangle$ were computed using the normalised

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consensus hydrophobicity scale of Eisenberg *et al.* [20]. These mean values were then used to determine VP1 candidacy for oblique orientated α-helix formation as described by Harris *et al.* [21] and further analysed using amphiphilicity profiling [22].

2.3 Whole lipid extract of *S. aureus* membranes

S. aureus, strain UL12 was grown in nutrient broth to the exponential phase (OD = 0.6; λ = 600 nm). 1 ml of culture was washed twice in Tris buffer (10 mM, pH 7.5) and centrifuged (15000 g, 5 min) to form a pellet. Whole lipid extract of membranes of *S. aureus*, strain UL12, were obtained using the procedure described by Bligh and Dyer [23]. The lipid extract was concentrated using a speed vac (Jouan) and stored at -20 ^{o}C under N_2.

2.4 Preparation of lipid unilamellar vesicles

Lipid unilamellar vesicles were prepared according to Keller *et al.*, [24]. Essentially, chloroform solutions of whole lipid extract of *S. aureus* membranes were dried with nitrogen gas and hydrated with HEPES buffer (10 mM, pH 7.5) to give final lipid concentrations of 150 mM. The resulting cloudy suspensions were sonicated at 4° C with a Soniprep 150 sonicator (amplitude 10 microns) until clear (30 cycles of 30 seconds), centrifuged (15 min, 3000 g, 4 °C) and the supernatant decanted for immediate use.

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2.5 FTIR conformational analysis of VP1

VP1 (1 mM) was solubilised in either: HEPES (10 mM, pH 7.5) or a suspension of vesicles formed from whole lipid extract of *S. aureus* membranes, prepared as described above with the overall lipid to VP1 ratio maintained at 50:1. These samples were spread individually on a CaF_2 crystal, and the free excess water evaporated at room temperature. The single band components of the VP1 amide I vibrational band (predominantly C=O stretch) were monitored using an FTIR '5-DX' spectrometer (Nicolet Instruments, Madison, WI, USA) and for each sample absorbance spectra were produced. Curve fitting was applied to overlapping bands using a modified version of the CURFIT procedure written by Dr Moffat, National Research Council, Ottowa, Canada, to determine the relative percentages of primary structure involved in secondary structure formation [25].

2.6 FTIR analysis of phospholipid phase transition properties

VP1 (1 mM) was solubilised in suspensions of vesicles formed from whole lipid extract of *S. aureus* membranes, prepared as described above with the overall lipid to VP1 ratio maintained at 50:1. As controls, corresponding vesicles were prepared with no peptide present. All samples were then subjected to automatic temperature scans with a heating rate of 3° C (5 min^{-1}) and within the temperature range 0° C to 60° C. For every 3°C interval, 50 interferograms were accumulated, apodized, Fourier transformed and converted to absorbance spectra. These spectra-monitored changes in the $\beta \longleftrightarrow \alpha$ acyl chain melting behaviour of phospholipids were determined as shifts in the peak position of the symmetric stretching vibration of the methylene groups, $\nu_s(CH_2)$, which is known to be a sensitive marker of lipid order [25].

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2.7 Monolayer studies on the lipid interactions of VP1

All experiments were conducted at a 21.0 ± 1 °C using a Langmuir Blodgett trough, NIMA Technology 601M (Coventry, UK). Monolayer studies were performed using a subphase containing either Tris buffer subphase (10 mM, pH 7.5.), or this buffer supplemented with 1 mM NaCl, which was continuously stirred by a magnetic bar (5 rpm). Surface tension was monitored by the Wilhelmy method using a Whatman's (Ch1) paper plate in conjunction with a microbalance [26].

The barriers of the Langmuir-Blodgett trough were adjusted to their maximum separation (surface area 80 cm^2) and this position maintained. To measure surface activity, VP1 was then injected into the buffer subphase to give final concentrations ranging between 1.0 and 30.0 µM and at each peptide concentration, changes in surface pressure at the air / water interface were monitored for 1 hour [27]. Maximal surface activity was observed at a VP1 subphase concentration of 20 µM, indicating that the air / water interface was saturated with peptide. This was taken as the optimum peptide concentration to study VP1 interactions with lipid monolayers and was used in all subsequent investigations.

The ability of VP1 to penetrate lipid monolayers at constant area was also studied. Monolayers were formed by spreading chloroform solutions of whole lipid extract of *S. aureus* membranes, which were prepared as described above onto a buffer subphase. The solvent was allowed to evaporate off over 30 minutes and then the monolayer compressed at a velocity of 5 cm^2 min^{-1} to give a surface pressure of 30 mN m^{-1}. The barriers were maintained in this position and peptide was then injected into the subphase to give a final peptide concentration of 20 µM. Interactions of the

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peptide with lipid monolayers were monitored as changes in monolayer surface pressure versus time.

3. Results and Discussion

Brandenburg *et al.,* [28] previously showed VP1 to adopt mainly β-type structures (92%) in aqueous solution using FTIR conformational analysis (Figure 1A [28]). However, VP1 was able to adopt an α-helical structure in the presence of lipid vesicles formed from dimyristolphosphatidylserine and dimyristolphosphatidylcholine with the levels of α-helicity remaining at *circa* 40% over lipid to peptide ratios ranging from 10:1 to 100:1 [18]. However, our analyses confirmed that VP1 was able to adopt comparable levels of α-helical structure (*circa* 45%) in the presence of vesicles formed from endogenous *S. aureus* membrane lipid (Figure. 1B). These data emphasise the importance of the amphiphilic environment provided by the membrane lipid interface in enabling VP1 to adopt an α-helical structure.

When VP1 is represented as a two-dimensional axial projection, the peptide is seen to form an amphiphilic α-helix with a cationic polar face and a wide apolar face, subtending an angle of 180° (Figure 2A). Hydrophobic moment analysis shows that this α-helix possesses levels of amphiphilicity ($\langle\mu H\rangle$ = 0.46) and hydrophobicity ($\langle H_0\rangle$ = 0.47) that are associated with membrane interactive oblique orientated α-helices [21]. Characteristic of this α-helical class, analysis of the VP1 α-helix using amphiphilicity profiling [22] revealed the presence of a hydrophobicity gradient, which increased along *circa* two thirds of the α-helical long axis in an N→C direction (Figure 2B). These theoretical data in combination with the previously published structural studies strongly support the prediction that VP1 would be able to form a

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membrane interactive oblique orientated α-helix comparable to those found in a range of antimicrobial peptides [14].

A bacterial toxicity assay [26] showed that VP1 exhibited an MLC of 3 mM against *S. aureus* with 100% lysis induced within 2 hours at this concentration implying it is therefore weakly antibacterial against Gram-positive organisms. The use of FTIR spectroscopy showed VP1 able to affect the lipid phase transition analysis of vesicles formed from endogenous *S. aureus* membrane lipid (Figure 3). In the absence of peptide, these vesicles underwent transition from the gel phase to liquid crystalline phase over the temperature range 30°C to 70°C with a concomitant increase in membrane fluidity, indicated by a rise in wavenumber from *circa* 2852.2 cm^{-1} - 2853.5 cm^{-1} (Figure 3). The presence of VP1 caused no apparent change to this temperature range but induced a significant increase in the fluidity as indicated by a shift up in wavenumber range to 2853.0 cm^{-1} - 2854.2 cm^{-1}, which is consistent with VP1 lipid interactions. These observations were reinforced by the results of Langmuir-Blodgett studies, which showed that VP1 partitioned into monolayers formed from endogenous *S. aureus* membrane lipid at an initial surface pressure characteristic of bacterial membranes (30 mN m^{-1}) causing maximal changes in monolayer surface pressure of 5.0 mN m^{-1} (Figure 4, curve A).

To elucidate the characteristics of VP1− *S. aureus* membrane interactions, the ability of the peptide to partition into a variety of pure lipid monolayers based on major components of the *S. aureus* membrane [29] was investigated. It can be seen from Figure 4 that VP1 induced maximal surface pressure changes of 4 mN m^{-1} over 7500 sec in monolayers formed from DMPE (curve B) but higher maximal surface pressure

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changes of 5.5 mN m^{-1} and at a threefold faster rate (2500 sec) in monolayers formed from DMPG (curve C). These results clearly suggest that VP1 has an affinity for DMPG / anionic lipid which may indicate a strong electrostatic contribution to VP1−membrane partitioning although there is likely to be an important hydrophobic contribution to partitioning due to the comparable levels of insertion seen with DMPE monolayers. The importance of the electrostatic component was confirmed by the observation that VP1 – DMPG interactions were reduced to insignificant levels in the presence of 1 mM NaCl (data not shown). This electrostatic contribution would be expected to involve the polar face of the VP1 α-helix with charge − charge interactions between arginine 5, the peptide's sole cationic residue, and anionic components of the membrane lipid headgroup region.

In conclusion, these combined FTIR and monolayer data clearly support the suggestion that VP1 has the ability to partition into bacterial membranes *via* the use of an amphiphilic oblique orientated α-helix. Based on the structural properties of this α-helix, it is suggested that the peptide would insert into the membrane at an angle such that its hydrophobic C-terminal region is able to interact with the membrane lipid acyl chain region. The data indicates a sequence with general amphiphilic properties, which as seen here has the ability to act as an antimicrobial, but the low toxicity levels would imply further sequence specific requirement for efficacy.

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Figure Legends

Figure 1 shows FTIR conformational analysis of VP1 in the presence of vesicles formed from whole lipid extracts of *S. aureus* membranes at a lipid to peptide ratio of 50:1. The numbers annotating spectra indicate peak band absorbencies. For each spectrum, the relative percentages of α-helical structure (1650 cm^{-1} - 1660 cm^{-1}) and β-sheet structures (1625 cm^{-1} - 1640 cm^{-1}) were computed, all as described in the methods. In aqueous solution, VP1 was formed from greater than 95% β-type structures (Figure 1A) but in the presence of vesicles with lipid compositions mimetic of *S. aureus* membranes (Figure 1B) the peptide was 45% α-helical.

Figure. 2. shows sequence analysis of VP1 adapted from Brandenburg *et al.,* [28]. Figure 2A. Two-dimensional axial projections of VP1 according to Schiffer *et al.,* [30]. Annotated numbers represent the relative locations of amino-acid residues within protein primary structure, and hydrophobic residues are circled. It can be seen that the α-helix possesses a glycine-rich polar face and a wide hydrophobic face rich in bulky amino-acid residues. Figure 2B shows the amphiphilicity profile, with increasing hydrophobicity along the α-helical axis in an N→C direction.

Figure. 3 shows FTIR lipid phase transition analyses of vesicles formed from whole lipid extracts of *S. aureus* membranes. In the absence of VP1 (□), these model membranes underwent a transition from the gel phase to the liquid crystalline phase over the temperature range 30° C to 70° C with a concomitant increase in membrane fluidity as indicated by the rise in wavenumber from *circa* 2852.0 cm^{-1} - 2853.5 cm^{-1}. The presence of VP1 caused no apparent shift in this temperature range but induced a significant increase in the fluidity of *S. aureus* membranes (▲), as indicated by a

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general rise in the wavenumber, which now moved from *circa* 2853.0 cm^{-1} - 2854.2 cm^{-1}.

Figure. 4 shows the time course of VP1 interaction with lipid monolayers. Monolayers were set at an initial surface pressure of 30 mN m^{-1}, mimetic of naturally occurring membranes, and VP1 was introduced into the Tris buffer subphase (10 mM, pH 7.5) of a Langmuir-Blodgett system at final concentration 20 µM, all as described in the methods section. For monolayers formed from whole lipid extract of *S. aureus* membranes (A), the peptide induced maximal surface pressure change of 5 mN m^{-1} over 1200 sec. However, the peptide induced maximal surface pressure changes of 4 mN m^{-1} over 7500 sec in DMPE monolayers (B) and 5.5 mN m^{-1} over 2000 sec in DMPG monolayers (C).

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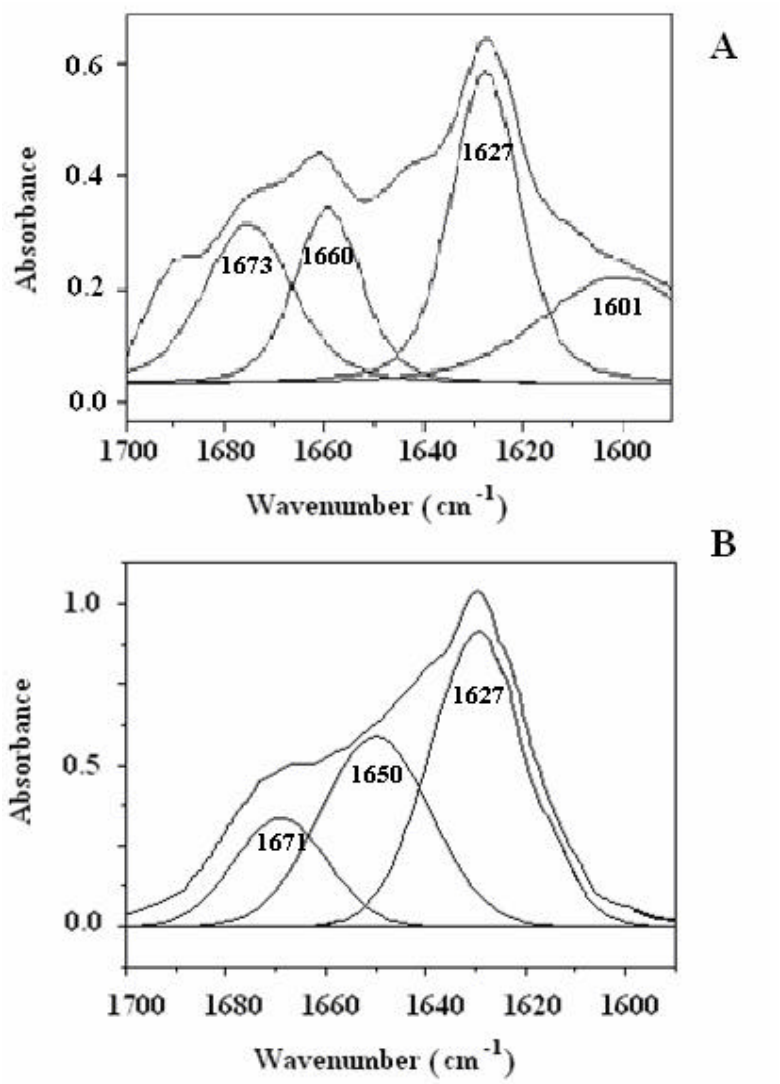

Figure 1

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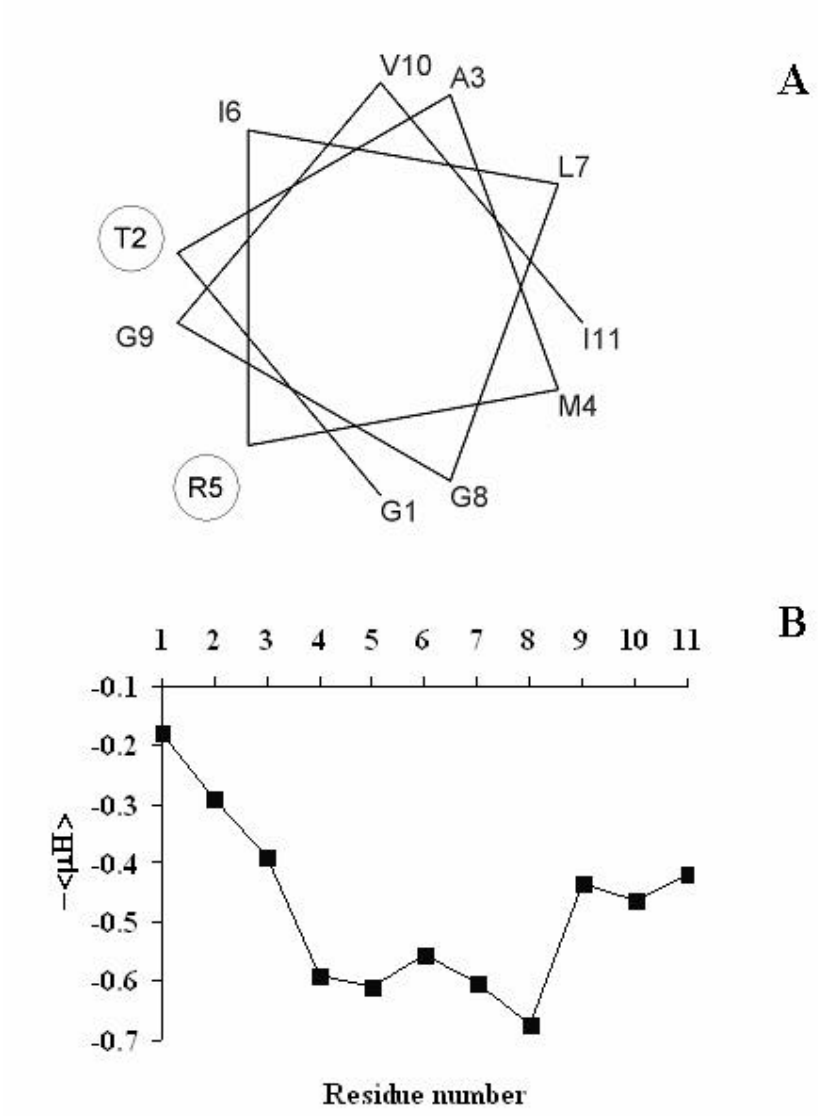

Figure. 2

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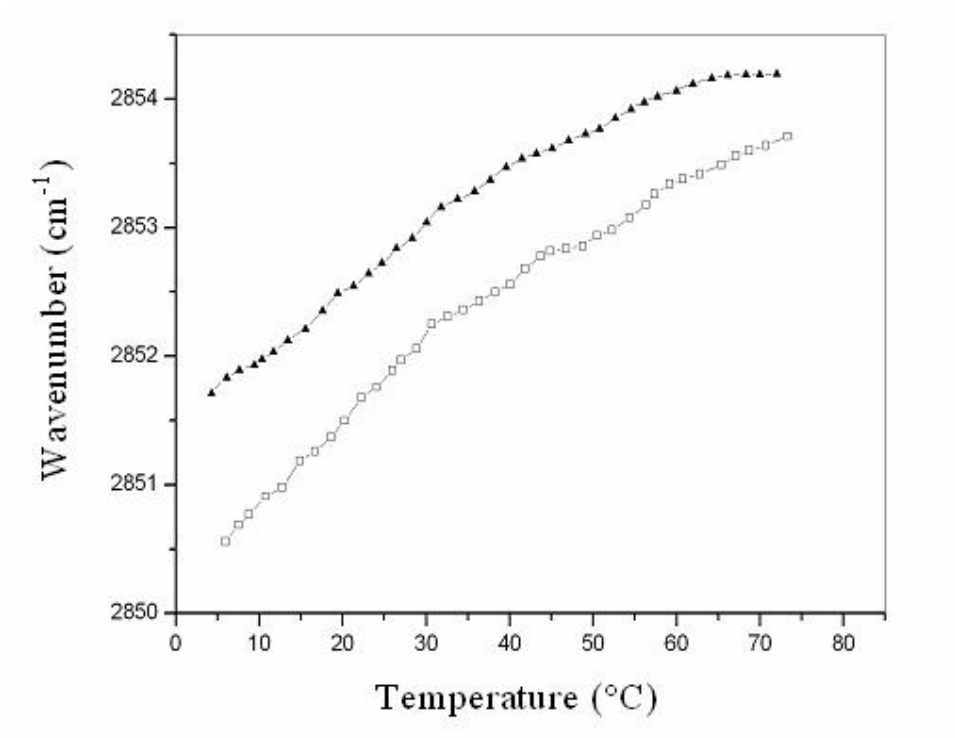

Figure 3

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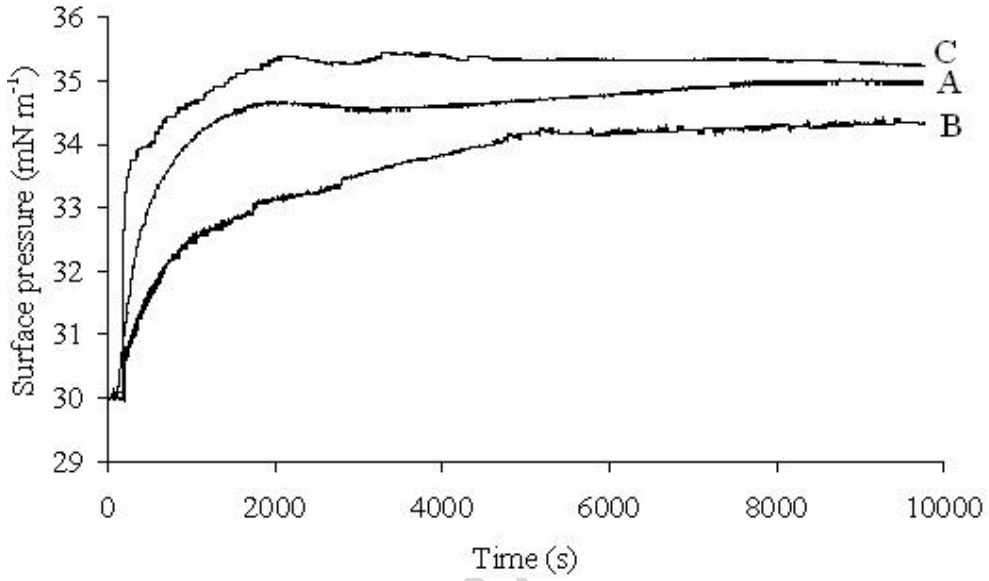

Figure 4

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