



รายงานวิจัยฉบับสมบูรณ์

โครงการการออกแบบเปปไทด์ที่มีสมบัติการสร้างรูร่วนผนังเซลล์

(*De Novo* Design of Pore-Forming Peptides)

โดย นายชชาติชาย กฤตนิยม

ตุลาคม 2544

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(De Novo Design of Pore-Forming Peptides)

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กิตติกรรมประกาศ

โครงการนี้ได้รับการทุนสนับสนุนจากสำนักงานกองทุนสนับสนุนการวิจัย (สกว.) ตามโครงการทุนวิจัยหลังปริญญาเอกประจำปี 2541 ตามสัญญาเลขที่ PDF/18/2541 การสนับสนุนด้านเครื่องมือและห้องปฏิบัติการจากสถาบันอณูชีววิทยาและพันธุศาสตร์ และสถาบันวิจัยและพัฒนาวิทยาศาสตร์และเทคโนโลยี มหาวิทยาลัยมหิดล

บทคัดย่อ

หมายเลขโครงการ PDF41/18/2541

ชื่อโครงการ การออกแบบเปปไทด์ที่มีสมบัติการสร้างรูวบนผนังเซลล์

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ที่ปรึกษาโครงการ

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ระยะเวลาการวิจัย กรกฎาคม 2541 – กรกฎาคม 2543 (ขยายเวลาถึงตุลาคม 2544)

วัตถุประสงค์

1. เพื่อออกแบบโครงสร้างสายเปปไทด์ ที่สามารถการสอดแทรกและก่อให้เกิดรูวบนผนังเซลล์ได้
2. เพื่อสังเคราะห์เปปไทด์ที่ก่อรูรั้วด้วยขบวนการสังเคราะห์ทางเคมีหรือด้วยวิธีการทางพันธุวิศวกรรม
3. เพื่อศึกษาปัจจัยพื้นฐานเกี่ยวกับการจัดตัวจากลำดับกรดอะมิโนไปสู่โครงสร้างที่มีหน้าที่เฉพาะ

ระเบียบวิธีการวิจัย

1. วิเคราะห์ลักษณะและทำแบบจำลองโครงสร้างของเปปไทด์ที่ก่อรูรั้ว
ศึกษาข้อมูลเบื้องต้นของโครงสร้างโปรตีนหลายชนิดที่ก่อรูรั้วบนผนังเซลล์ เพื่อวิเคราะห์ลักษณะที่เอื้อต่อการทำหน้าที่ ทำการสร้างแบบจำลองโครงสร้างสามมิติที่คาดหวังและทดสอบทางด้านเสถียรภาพ
2. สังเคราะห์เปปไทด์และตรวจสอบโครงสร้างเทียบกับแบบจำลอง
ทำการสังเคราะห์เปปไทด์ขึ้นด้วยวิธีการทางเคมี แยกบริสุทธิ์ด้วยโครมาโตกราฟี และทดสอบค่ามวลโมเลกุล จากนั้นทำการวิเคราะห์โครงสร้างทุติยภูมิเปรียบเทียบกับแบบจำลอง
3. ทดสอบคุณสมบัติการก่อรูรั้วบนผนังเซลล์สังเคราะห์
ตรวจสอบการสอดแทรกกับชั้นไขมันด้วยลิโปโซม และทดสอบการสร้างรูรั้วบนผนังเซลล์สังเคราะห์ด้วยเครื่องทดสอบศักย์ไฟฟ้าของเซลล์เมมเบรน

ผลการวิจัย

1. แบบจำลองจำนวนหนึ่งได้ถูกสร้างขึ้น โดยอ้างอิงกับโครงสร้างแบบเกลียวอัลฟา ในการออกแบบแบบจำลองเหล่านี้ กำหนดให้มีการใช้กรดอะมิโนที่พบมากในโครงสร้างแบบอัลฟา และมีการจัดลำดับเพื่อให้มีการแยกส่วนของโครงสร้างบริเวณที่ชอบและไม่ชอบน้ำต่างกัน ผลการทดสอบค่าพลังงานของแบบจำลองพบว่า มีความเสถียรเพียงพอที่จะจัดโครงสร้างได้ตามที่คาดหมายไว้
2. สายโพลีเปปไทด์จำนวน 4 แบบได้ถูกสังเคราะห์ขึ้นตามลำดับกรดอะมิโนของแบบจำลอง สามารถแยกบริสุทธิ์ได้ด้วยโครมาโตกราฟี และได้รับการตรวจสอบความถูกต้องด้วยการวิเคราะห์มวลโมเลกุลจากการทดลองวิเคราะห์โครงสร้างพบว่า สายโพลีเปปไทด์ทั้ง 4 ชนิดมีโครงสร้างแบบเกลียวอัลฟาตามที่ได้คาดหมายไว้เมื่ออยู่ในสารละลายที่มีขั้วต่ำ
3. ในการทดสอบการสอดแทรกกับชั้นไขมันของลิโปโซมพบว่า สายเปปไทด์แบบ PEP-4 เพียงชนิดเดียวที่สามารถสอดแทรกกับชั้นไขมันได้ รวมทั้งยังเป็นสายเปปไทด์เพียงชนิดเดียวที่สามารถก่อรูรั่วนบนผนังเซลล์สังเคราะห์ได้ รูรั่วที่สร้างขึ้นจากเปปไทด์ดังกล่าวเป็นชนิดที่เลือกผ่านเฉพาะไอออนบวก มีค่าการนำไฟฟ้าประมาณ 188 พิโคซีเมนส์

สรุปสาระสำคัญของผลที่ได้จากการวิจัย

ผลการทดลองในโครงการนี้ได้แสดงให้เห็นถึงความเป็นไปได้ในการออกแบบและสังเคราะห์สายโพลีเปปไทด์ให้มีคุณสมบัติและหน้าที่การทำงานตามที่ต้องการได้ โดยเปปไทด์ที่สร้างขึ้นมิได้มีลำดับกรดอะมิโนเหมือนกับที่มีอยู่แล้วตามธรรมชาติ (*de novo approach*) เปปไทด์ที่พบว่าสามารถก่อรูรั่วนบนผนังเซลล์ได้ในการทดลองนี้มีความยาวเท่ากับ 16 ลำดับกรดอะมิโน มีการเรียงตัวของกรดอะมิโนที่ทำให้ผิวของโครงสร้างเกลียวอัลฟามีความเป็นขั้วประมาณ 30% และอยู่รวมตัวกันเพียงด้านหนึ่ง และพื้นผิวที่เหลือทั้งหมดเป็นพื้นที่ไม่ชอบน้ำ โดยรูรั่วที่เกิดขึ้นโดยเปปไทด์ดังกล่าวเป็นชนิดเลือกผ่านเฉพาะไอออนบวก มีค่าการนำไฟฟ้าประมาณ 188 พิโคซีเมนส์

ข้อเสนอแนะสำหรับการวิจัยในอนาคต

การศึกษาวิจัยในอนาคตควรเป็นการศึกษากลไกของการสอดแทรกและก่อรูรั่วว่ามีรายละเอียดอย่างไร หรือรูรั่วที่เกิดขึ้นมีลักษณะโครงสร้างอย่างไร ข้อมูลเพิ่มเติมเหล่านี้จะเป็นประโยชน์ต่อการออกแบบและปรับปรุงการทำหน้าที่ให้ดีขึ้นได้ต่อไป

คำสำคัญ

การออกแบบเปปไทด์ โครงสร้างเกลียวอัลฟา การก่อรูรั่วนบนผนังเซลล์

Abstract

Project Code: PDF41/18/2541

Project Title: *De Novo* Design of Pore-Forming Peptides

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Project Period: July 1998 – July 2000 (with extension to October 2001)

Objectives:

1. To de novo design a synthetic peptide with pore-forming activity
2. To synthesize the designed peptides using chemical or genetic methods
3. To study the concept of structural folding from a linear sequence to functional molecule

Methodology:

1. Structural analysis and computer modeling of pore-forming peptides
2. Peptide synthesis and structural characterization
3. Assay of pore-forming activity on artificial membrane

Results:

1. A numbers of peptide sequences were designed with expected helical conformation. The amino acids selected were among those with high helical propensity. The amphipathic characteristics on the helix contain hydrophilic and hydrophobic residues of approximately 30 and 70% respectively. Energy minimization of these peptide models gave a comparable stability with a known helical peptide.
2. We have 4 peptides synthesized according to the sequences of selected models using solid-phase synthesis. The purified products were obtained from reverse-phase chromatography and conformed for their correct sequences by mass spectrometry. Structural characterization of these peptides in organic solvent using circular dichroism revealed the helical conformation.

3. Study for peptide interaction with liposome demonstrated that only PEP-4 peptide was able to release the entrapped glucose from the liposome. Further assay of this peptide showed an ion channel activity on the planar lipid bilayer system. The channel formed by PEP-4 peptide is suggested to be a cation selective with conductance of 188 pS.

Discussion Conclusion:

This work has demonstrated that a *de novo* of synthetic peptides for specific function could be accomplished using the basic concept of protein structure. Our synthetic pore-forming peptide (PEP-4) is 16 residues in length containing approximately 30% of hydrophilic surface on a single side of the helical conformation. The channel of this peptide on an artificial membrane suggested a cation selective property with conductance of 188 pS.

Suggestions:

The future study of this pore-forming peptide should be focused on the pore-forming mechanism and the structure of the pore. This information would assist in the design strategy for pore-forming peptide with improved activity.

Keywords:

Peptide design , helical conformation, membrane pore formation

ส่วนที่ทำหน้าที่เกี่ยวข้องกับการสร้างรูวบนผนังเยื่อหุ้มเซลล์ โดยส่วนโครงสร้างที่ก่อรูวนี้จำแนกได้เป็นสองกลุ่มใหญ่คือ กลุ่มที่มีโครงสร้างแบบเกลียวอัลฟา(α -helix)และกลุ่มที่มีโครงสร้างแบบแถบเบตา(β -strand) โครงสร้างแบบเกลียวอัลฟานั้นพบในโปรตีน colicin A (Parker et al., 1989), colicin Ia (Wiener et al., 1997), colicin E1 (Elkins et al., 1997), diphtheria toxin (Choe et al., 1992), δ -endotoxin ชนิด Cry1Aa (Grochulski et al., 1995) และชนิด Cry3A (Li et al., 1991) และโครงสร้างแบบเบตานั้นพบในโปรตีน α -hemolysin (Song et al., 1996), anthrax toxin (Petosa et al., 1997) และ δ -endotoxin ชนิด CytB (Li et al., 1996)

กลุ่มวิจัยของเรามีความสนใจหลักในโครงสร้างแบบเกลียวอัลฟา โดยลักษณะของเกลียวอัลฟาที่พบมักมีคุณสมบัติของ amphipathicity ที่เอื้อต่อการจัดเรียงและรวมตัวอย่างมีรูปแบบเฉพาะ เพื่อสร้างให้เกิดรูวบนผนังเซลล์ เช่นรูวที่เกิดจาก colicin A เกิดขึ้นจากกลุ่มเกลียวอัลฟาจำนวน 10 เกลียวรวมกัน (Parker et al., 1989, 1992) ส่วนรูวที่เกิดจาก diphtheria toxin และ Cry δ -endotoxin นั้น ได้มีการเสนอแบบจำลองของรูวที่เกี่ยวข้องกับการจัดเรียงตัวกันของเกลียวอัลฟาในลักษณะคล้ายคลึงกัน (Cummings et al., 1994; Gazit & Shai, 1995; Parker & Pattus, 1993; Li et al., 1991; Silverman et al., 1994)

งานวิจัยทางด้านวิศวกรรมโปรตีนในปัจจุบัน สามารถออกแบบและสังเคราะห์โครงสร้างเปปไทด์หรือโปรตีนให้มีโครงสร้างและหน้าที่ตามความคาดหวังต่างได้ ทั้งโดยการเลียนแบบธรรมชาติด้วยการคงไว้เฉพาะส่วนสำคัญ(protein minimization) และการทำขึ้นใหม่ (specific design) เช่นการสังเคราะห์โครงสร้างเดี่ยวแบบอัลฟา(α) หรือเบต้า(β) โครงสร้างแบบผสมอัลฟา-อัลฟา(α -turn- α) (Dolphin & Baltzer, 1997) โครงสร้างแบบกลุ่มเกลียวอัลฟา (α -helix bundle) (Dieckmann & DeGrado., 1997; Fairman et al., 1996; Predki & Regan, 1995) หรือโครงสร้างแบบเกลียวซ้อน (coiled coils) (Lumb & Kim, 1995) โดยในการออกแบบโครงสร้างเหล่านี้จำเป็นต้องมีความรู้ความเข้าใจในหลักการด้านต่างๆ เช่น การจัดให้มีปฏิสัมพันธ์ที่เหมาะสมทั้งในระยะใกล้และไกล (Blanco et al., 1997; Gurunath et al., 1995; Stellwagen et al., 1992) การกำหนดขนาดของ loop ที่เหมาะสม (Nagi & Regan, 1997) การวิเคราะห์ทางเสถียรภาพ(Chmielewski & Lipton, 1994) และการเชื่อมต่อทางเคมีเพื่อให้เกิดโครงสร้างที่ใหญ่ขึ้น (You et al., 1996)

โครงการวิจัยนี้เป็นการรวบรวมความรู้เกี่ยวกับความสามารถในออกแบบโครงสร้างของโปรตีนและเปปไทด์เข้ากับความรู้พื้นฐานเกี่ยวกับโปรตีนที่ก่อรูวจากแบคทีเรีย เพื่อออกแบบและสร้างโมเลกุลเปปไทด์ที่มีโครงสร้างแบบอัลฟาและมีสมบัติในการก่อรูวในห้องปฏิบัติการ เนื่องจากงานวิจัยพื้นฐานในลักษณะนี้ยังมิได้เกิดขึ้นอย่างแพร่หลายภายในประเทศ โครงการวิจัยนี้จึงเสมือนเป็นจุดเริ่มต้นที่สำคัญในการสร้างองค์ความรู้และพัฒนาความรู้ความเข้าใจในหลักการที่เกี่ยวข้องกับการออกแบบ การสังเคราะห์ และการวิเคราะห์ทางโครงสร้างเปปไทด์ ตลอดจนการวิเคราะห์ปฏิสัมพันธ์ในการก่อรูวบนผนังเซลล์สังเคราะห์ให้กับนักวิจัยไทย อันจะเป็นประโยชน์ต่อวงการวิจัยพื้นฐานต่อไป

วัตถุประสงค์

1. เพื่อศึกษาออกแบบโครงสร้างสามมิติของเปปไทด์ ที่มีสมบัติในการสอดแทรกและก่อรูรั่วบนผนังเซลล์
2. เพื่อสังเคราะห์โครงสร้างเปปไทด์ที่ก่อรูรั่วด้วยขบวนการสังเคราะห์ทางเคมี หรือโดยพันธุวิศวกรรม
3. เพื่อศึกษาปัจจัยพื้นฐานในการจัดตัวจากลำดับกรดอะมิโนไปสู่โครงสร้างสามมิติที่มีหน้าที่เฉพาะ

ระเบียบวิธีวิจัย

การวิเคราะห์ลักษณะและทำแบบจำลองโครงสร้างของเปปไทด์ที่ก่อรูรั่ว

(Structural Analysis and Computer Modeling of Pore-Forming Peptides)

ทำการศึกษาข้อมูลเบื้องต้นของโครงสร้างโปรตีนต่างๆที่ก่อรูรั่วบนผนังเซลล์ เพื่อรวบรวมคุณลักษณะที่เอื้อต่อการสอดแทรกและก่อรูรั่วเข้าไปในโครงสร้างเหล่านี้ การวิเคราะห์โครงสร้างโดยคอมพิวเตอร์สมรรถนะสูงแบบSilicon Graphics Workstation เพื่อข้อมูลในเชิงปริมาณสำหรับการสร้างแบบจำลองสามมิติ การวิเคราะห์และสร้างแบบจำลองที่จะกระทำโดยชุดโปรแกรมสำเร็จรูป Insight II โดยแบบจำลองโครงสร้างที่สร้างขึ้นจะถูกทดสอบทางด้านเสถียรภาพ เพื่อคัดเลือกโครงสร้างที่เหมาะสมในการสังเคราะห์ต่อไป

การสร้างเปปไทด์และตรวจสอบโครงสร้างเปรียบเทียบกับแบบจำลอง

(Synthesis and Structural Characterization of Designed Peptides)

จากลำดับกรดอะมิโนของแบบจำลองสามมิติที่สร้างขึ้นจากคอมพิวเตอร์ สายของเปปไทด์จะถูกสังเคราะห์ขึ้นในห้องปฏิบัติการด้วยขบวนการทางเคมีแบบ Solid Phase Peptide Synthesis (SPPS) ทำการแยกบริสุทธิ์ด้วยเทคนิคโครมาโตกราฟี และยืนยันความถูกต้องของลำดับกรดอะมิโนด้วยการทดสอบมวลโมเลกุล สายโพลีเปปไทด์ที่ได้จากการสังเคราะห์จะถูกวิเคราะห์หาโครงสร้างทุติยภูมิ (secondary structure) ด้วยเทคนิค Circular Dichroism Spectroscopy การตรวจสอบสมบัติเชิงโครงสร้างนี้จะเป็นข้อมูลยืนยันว่าโครงสร้างที่สร้างขึ้นว่ามีลักษณะตามที่คาดหมายไว้หรือไม่

การศึกษาและทดสอบสมบัติการก่อรูรั่วบนผนังเซลล์สังเคราะห์

(Study of Pore Formation on Artificial Membrane)

ทำการศึกษาทดสอบเกี่ยวกับปฏิสัมพันธ์ของเปปไทด์ที่มีต่อผนังเยื่อหุ้มเซลล์ ด้วยเทคนิค liposome entrapped release assay (Kinsky, 1974) เพื่อทดสอบความสามารถเบื้องต้นในการก่อรูรั่วของเปปไทด์ โดยเป็นการตรวจวัดปริมาณการรั่วไหลของโมเลกุลกลูโคสที่ถูกปล่อยออกจากหน่วยของ liposome เมื่อเกิดรูรั่วขึ้น การทดสอบอย่างละเอียดในขั้นต่อไปกระทำโดยเทคนิค planar lipid bilayers (Schwartz et al., 1993) เพื่อให้ได้ข้อมูลในเชิงคุณภาพและเชิงปริมาณ อันจะช่วยให้ทราบได้ถึงสมบัติในการความแยกแยะของรูรั่วต่อโมเลกุลสาร และสมบัติเฉพาะของรูรั่วที่เกิดขึ้นต่อไป

เนื้อหาสรุปของงานวิจัย

Introduction

The *de novo* design of peptide and protein has been an attractive approach for studying of the structure and function of proteins. The basic concept of *de novo* design involves a planing and construction of a protein or polypeptide, with expectation for folding of the linear sequence into a defined three dimensional structure. It has been demonstrated in a growing number of cases that the designed processes had accomplished their goals, leading to the protein and peptide products with precise structure and function. In the last several years, the study and characterization of protein secondary structure, especially in the helical conformation has revealed significant understanding of how the structure is formed and stabilized in nature. The knowledge accumulated from both theoretical and experimental works has currently led to a challenge among researchers in making a custom design for any desired structure and functions.

Our previous experience in construction and characterization of helical peptides revealed that the selection of amino acid residues according to their helical propensity might not be sufficient for the design of helical structures. There are several other factors to be considered closely, for example; the local interaction and stabilization among the neighboring residue , buffer or solvent environment (Krittanaei *et. al.*, 2001) as well as conformational entropy (Aurora *et. al.*, 1997)

This work aims to make a designed structure for a helical peptide using a compiled knowledge of protein secondary structure. The constructed peptide product is predicted to adopt not only a defined structure but also a unique function in pore-forming activity across a membrane. The results of this work would provide another exercise case for a possibility for the *de novo* design of structure for desired function.

According to the results obtained from this work, we have designed a group of amino acid sequences based on helix formation concept. After model and energy minimization analysis those peptides were chemically synthesized and chromatographic purified. Circular dichroism spectroscopy revealed a helical structure of our peptides in organic solvent. Characterization for pore-forming activity for the constructed peptides using small unilamellar vesicle (SUV) or liposome was found positive in one peptide sequence (PEP-4). This peptide was assayed further and found to form an ion channel on the artificial membrane. The resulting channels are cation selective with conductance around 190 pS. This work has demonstrated that the design of peptide sequence from scratch can be accomplished from the basic design concept yielding a peptide structure of desired function.

Materials and Methods

Materials and Methods

Step II. Washing:

- The protecting group was washed away with 10 ml-DMF twice, 5-minutes for each washing with 300-RPM shaking.

Step III. Coupling:

- Weigh out amino acid and solubilized in the HOBT/TBTU solution to reach equivalence ratio of 1.0 : 1.0 : 0.95, AA: HOBT:TBTU
- 1.5 equivalence of DIPEA was added into the bottle and then immediately the soluble amino acid cocktail (AA/HOBT/TBTU).
- Shaking for 30 minutes

Step IV. Washing:

- Uncoupling amino acid and coupling reagents from the reaction were washed away with 10 ml DMF. Three washings of 5 minutes each.

The cycle was repeated by changing amino acid in step III according to the peptide sequence from C to N-terminal. The final peptide product bound to the resin was removed to a free peptide through a cleavage reaction using 10 ml of 95% trifluoroacetic acid (TFA) in double distilled water. The peptide was filtered on a fritter glass funnel and precipitated with ice-cold diethyl ether.

Purification of Synthetic Peptides

Crude peptides obtained from solid phase synthesis were solubilized in 50% acetonitrile/ MilliQ water. After filtration with membrane (0.45- μ m pore size), samples were injected into an AKTApurifier liquid chromatography system (Amersham Pharmacia Biotech). The system was equipped with a reverse phase PepRPC 10/10 column. Elution was performed by generating a buffer gradient between two mobile phases: A, 0.1% TFA in water and B, 0.1% trifluoroethanol in acetonitrile. The linear gradient from 10% to 85%B was programmed within 20 min 1.5 ml/min flow rate. Fractions were collected for every 1 ml corresponding to UV absorption at 280 nm. Purified peptides were then lyophilized into solid form using a MAXI-LYO lyophilizer.

Mass Spectrometry

Molecular mass of the synthetic peptides were analyzed by Electro-spray Ionization Mass Spectrometry (ESI-MS) using an API-365 LC/MS/MS triple quadrupole mass spectrometer (PE SCIEX, USA). The instrument was equipped with an atmospheric pressure ionization source. Polypeptide samples were dissolved in methanol/ acetonitrile (1:1, v/v) and transferred into an ionspray needle using a 100- μ l glass syringe driven by automatic plunger. The infusion rate was set at 5 μ l/minute. The ionization potential was optimized in a range of 4800 – 5200 volts. Q1 mass analyzer was scanned from 200 to 2000 m/z units. An orifice potential was varied from 20 – 120 electron volts and electron multiplier detector was set at 2200 electron volts.

Determination of Peptide Concentration

Peptide concentrations were determined from an aromatic absorption at 280 nm. Purified peptides in sodium phosphate buffer pH 9.0 were loaded into a suprasil cuvette of 0.01 cm pathlength and scanned from 260 to 320 nm on a Cary-300Bio UV-Vis

spectrophotometer. A known extinction coefficient of tyrosine ($1,280 \text{ cm}^{-1}\text{M}^{-1}$) was used for all calculations.

Circular Dichroism Spectroscopy

Secondary structure of synthetic peptides was characterized using a JASCO J-715 spectropolarimeter (JASCO Inc.). The instrument was purged with oxygen free nitrogen (OFN) at 15 L/min before and during measurements. Purified samples were prepared in sodium phosphate buffer pH 7.20 and in 50% acetonitrile (ACN) at 1-10 mM concentration. Spectra were obtained in the far UV region from 190 to 260 nm at 20 nm/minute scanning rate, 2 second response, 2 nm bandwidth, and 3-5 accumulations. Digitized data were exported and plotted on Sigma plot.

Liposome Entrapped Glucose Release Assay

Liposomes with entrapped glucose were prepared using a modified method of Kinsky (1974). A lipid mixture of 12.5 umole phosphatidylcholine (PC), 3.6 umol dicetyl phosphate, and 1.8 umol of cholesterol in 3 ml chloroform/methanol (2:1, v/v) was added into a round bottomed flask. The solvent was evaporated out under vacuum at 37 C. The resulting lipid film was then resuspended in 0.5 ml of 300 mM glucose, 10 mM HEPES, pH 8.0. Small unilamellar vesicles (SUVs) were prepared by squeezing the suspension through the extruder membrane (0.1 um diameter, from Avanti Polar Lipid, USA) for a minimum of 11 passes. Unentrapped glucose was removed from the SUV suspension by gel filtration chromatography using a PD-10 column (Sephadex G-25, Pharmacia, Uppsala, Sweden). The column was equilibrated with 150 mM KCl, 10 mM HEPES, pH 8.0. An aliquot of washed liposomes (100 nmole of PC) was loaded into a 1 ml- disposable polymethyl methacrylate cuvette (Brand, Germany) containing 1 unit of hexokinase (Sigma), 1 unit of glucose-6-phosphate dehydrogenase (Sigma), 1 mM ATP, 0.5 mM NADP, 2 mM $\text{Mg}(\text{OAc})_2$ in 150 mM KCl, 10 mM HEPES, pH 8.0. Glucose release was monitored at 25 C for an increased absorbance for NADPH at 340 nm using a HP 8453 UV-Vis spectrophotometer (Hewlett Packard, USA). The relative glucose-release activities were indicated as a fraction of the maximum release, which is defined as the amount released after adding 0.1% Triton X-100.

Ion Channel Assay by Planar Lipid Bilayer System

A planar lipid bilayer system was performed using an Axopatch-1D patch-clamping system (Axon Instruments, USA). The CV-4B-0.1/100U Headstage ($50\text{M}\Omega$ - $50\text{G}\Omega$ resistance) was used for voltage activation and detection on the artificial membrane. Reaction chamber were shielded inside a Faraday's case and grounded for noise reduction. Artificial membrane was prepared fresh for each experiment from a mixture of phosphatidyl ethanolamine (PE), phosphatidyl chloride (PC), and cholesterol (CH) at 7:2:1 ratio. An aperture (200 μm diameter) on the Teflon reaction cup was pretreated with lipid mixture and dried under nitrogen gas purging. The cup was placed in the reaction chambers and filled with 150 mM KCl, 10 mM Tris, 1 mM CaCl_2 , pH 8.0. After the aperture was painted with lipid mixture, the baseline signal was monitored for membrane stability at least 30-45 minutes and then the current was recorded at command potential 0.0 mV. 5-10 μg of purified peptides were added into *cis* chamber and induced for membrane incorporation by changing command potentials up and down. Once square jumps of current were detected, the data were then recorded by applying various command potentials from -100 to +100 mV.

Results and Discussion

Peptide Design

Structural analysis of the pore-forming peptides and protein fragments from natural sequences within our data set has reveal a numbers of their common characteristics. Those observations may be list as follow:

- Their helical conformations are made up with a fair amount of amino acid residues of high helical propensity such as alanine, leucine, and glutamate.
- An amphipathic pattern is found lining through their helical surfaces. The hydrophobic area is dominated or at least equally presented on the helix.
- Their helical length is normally 20 or more amino acid residues for membrane translocation.
- Polar and charged residue are present for assisting in solubility in buffer solutions.
- Conformational stabilization found in some examples is located between residue i and $i+4$ on their sequences.
- Aromatic residues are sometime found near the N-terminal of the sequences, possible to interact with lipid membrane.
- A few polar or charge could be found inside the hydrophobic area for solubilization.

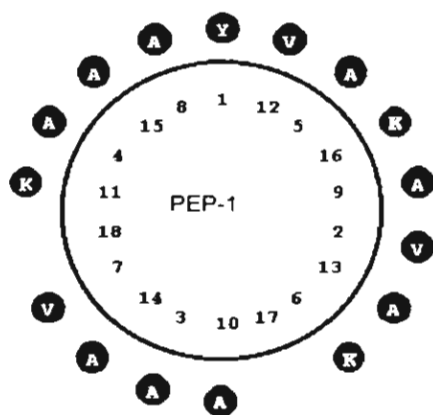
Our peptides were design to adopt 16 amino acid in their lengths, which are around 4-5 turns or 26 Angstrom around the helix axis. This length is slightly shorter than the thickness of most membrane. The construction of relative hydrophobic and hydrophilic surface ratio on the amphipathic pattern was varied from 30 to 50% of hydrophilic surface areas. Choices of polar and charge residues were set for small polar side-chains for good intermolecular packing among the peptide on the pore structure.

All of our sequences contain a tyrosine residue on the N-terminal based on the enhancement of peptide-lipid interaction. Our selected design sequences are shown in Table 1. The helical wheel representation of these designed peptides is depicted in Figure 1 and they were subjected to their model construction in further steps.

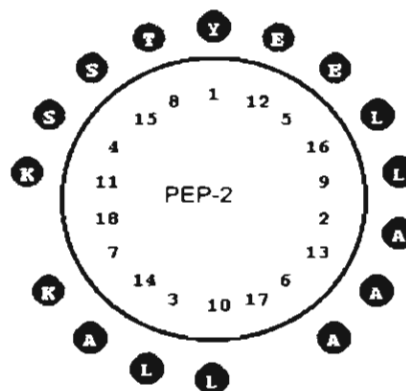
Peptides	Sequences
PEP-1	Ac-Y-VAAAK-VAAAK-VAAAK-OH
PEP-2	Ac-Y-ALSEA-KTLLK-EAASL-OH
PEP-3	Ac-Y-KALAE-ALARA-LADSA-OH
PEP-4	Ac-Y-ALSLA-ATLLK-EAASL-OH

Table 1: Selected sequences of the designed peptide with assigned names

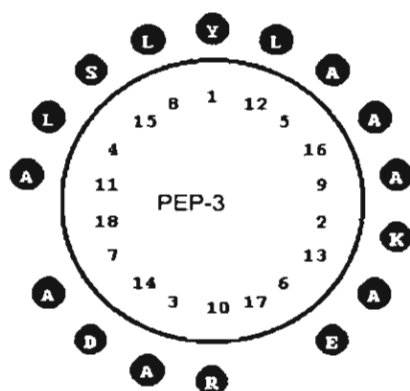
C:\CK\PDB\PEP1.SEQ
YVAAAKVAAAKVAAAK



C:\CK\PDB\PEP2.SEQ
YALSEAETLLKEAASL



C:\CK\PDB\PEP3.SEQ
YKALAFALAPALADSA



C:\CK\PDB\PEP4.SEQ
YALSLAATLLKEAASL

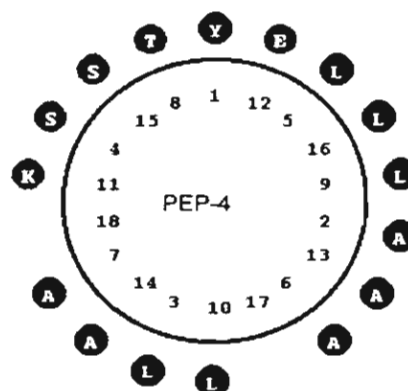


Figure 1: Helical wheel of the amphipathic peptide models showing the distribution of hydrophobic (in black) and hydrophilic (in gray) residues along the helical surface.

Molecular Modeling

Energy minimization performed on Discover module of InsightII showed that the initial energies for all PEP-1, PEP-2, PEP-3 and PEP-4 molecules were decreased dramatically after the minimization. The total energy reduction was found as the results of decreased potential from both Van de Waals (vdw) and electrostatic interactions. The number of iterations was set for 100 iterations for each run and continued until the total energy become stable (400-500 iterations). The initial and minimized energy is listed in Table 2.

	PEP-1	PEP-2	PEP-3	PEP-4
<i>Initial States</i>				
▪ Van de Waals	252.995	796.608	515.927	566.482
▪ Electrostatic	162.519	85.385	-67.399	83.315
▪ Total	415.514	881.993	448.528	649.797
<i>Minimized State</i>				
▪ Van de Waals	79.232	79.000	74.276	77.726
▪ Electrostatic	137.613	60.524	-93.865	55.481
▪ Total	216.845	139.524	-19.589	133.207

Table 2: Initial and minimized energy of peptide models from steepest descents algorithm.

From the designed sequences of our peptides, we have used the energy minimization as a mean to determine whether the molecule can adopt a stable conformation with reasonable energy. The conformation that we employed for this calculation was alpha helical with standard bond length and angles (Figure 2). Our previous data on PEP-1 characterization has demonstrated that the peptide is in a stable helical conformation (Krittanaï and Johnson, 2000). The energy of PEP-2, PEP-3 and PEP-4 obtained from our calculation may be viewed as arbitrary parameters relative to PEP-1. Their total energy in this work were lower than that of PEP-1 suggesting that they were able to adopt a helical conformation with good stability. Our data for PEP-3 showed a marked drop of the total energy. This is possibly caused by the distribution of charge and polar side-chains on the molecule that provide good electrostatic stabilization to the peptide. According to these energetic results, we may expect that all of our designed sequences are capable of adopting a helical conformation with good stability as they were planned from the designing step. However this prediction will be proofed further in structural characterization

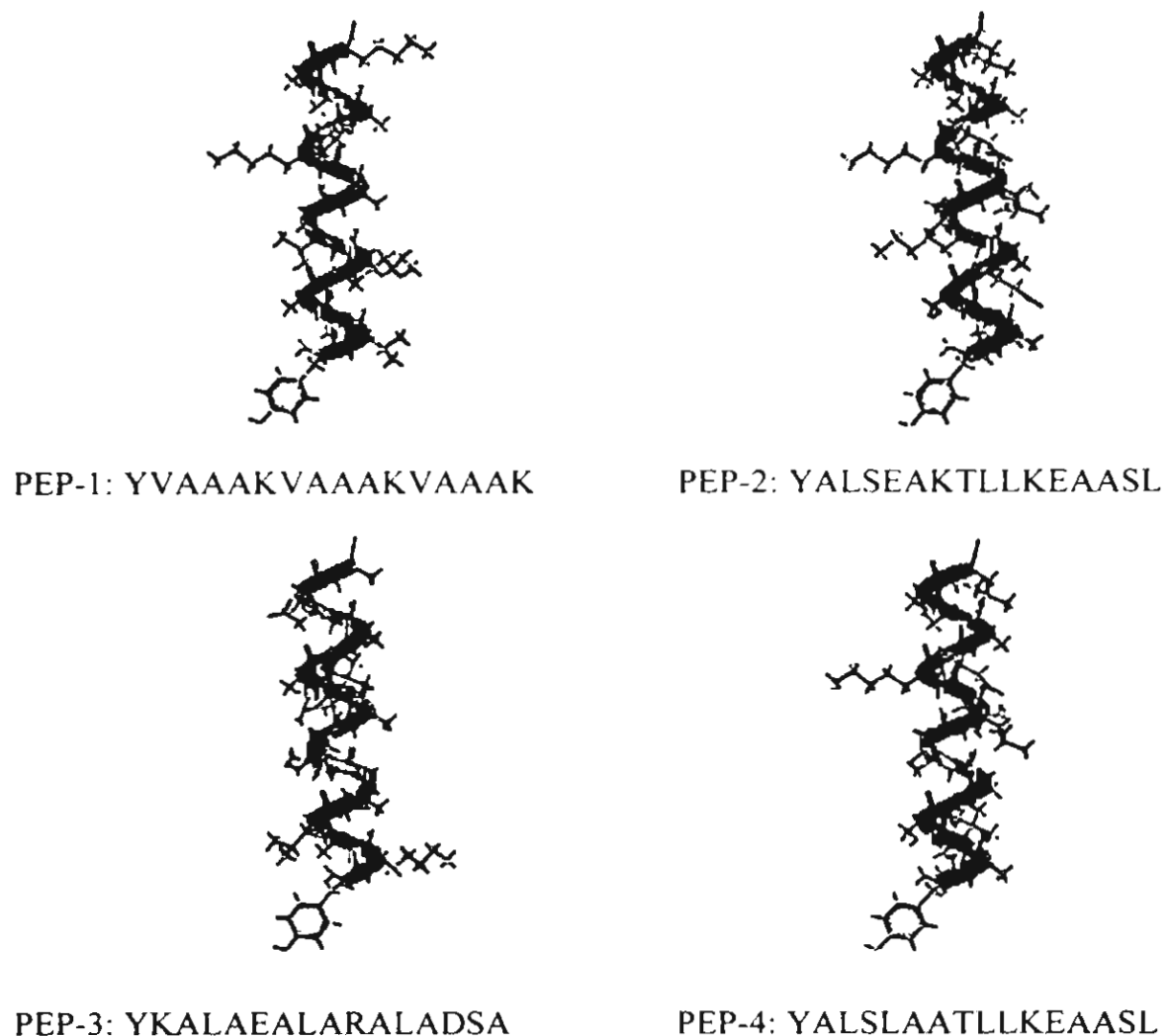


Figure 2: Structural representations of peptide models constructed from standard parameters of alpha-helical conformation.



Solid Phase Peptide Synthesis

From the solid phase peptide synthesis we were able to get peptide products as expected. All of the peptides obtained after ether precipitation were observed as a white powder and the product yield were determined after drying a fume hood for overnight. Their yields were found 60-70% of the expected molar. Solubility test of these peptides showed moderate solubility in aqueous buffers and 50% acetonitrile.

Purification of Synthetic Peptides

Reverse phase chromatography has been proven to be an effective mean for peptide purification since it is fast and simple. We injected our solubilized crude peptides into the hydrophobic column media at equilibrium in 10% acetonitrile. PEP-1 was found to eluted out earlier than other peptides while PEP-4 was found to be the latest (Figure 3 - 6). This can be explained based on differences in hydrophobicity of the peptides. All of the purified peptide fractions were identified in the major peaks of chromatogram, as an indication for an existence as a major product from synthesis.

The collected fraction of purified peptides was lyophilized for solvent removal and the products were obtained as a puffy powder form. They were stored in a – 40 °C long-term freezer and stock solutions were prepared fresh for each experiment.

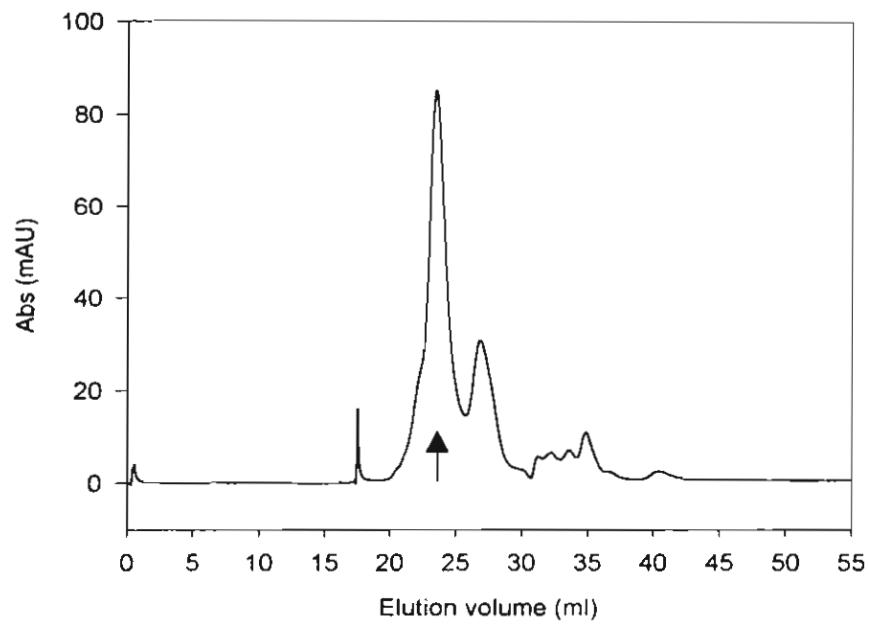


Figure 3: Chromatogram of PEP-1 purification. The purified fraction is labeled with arrow.

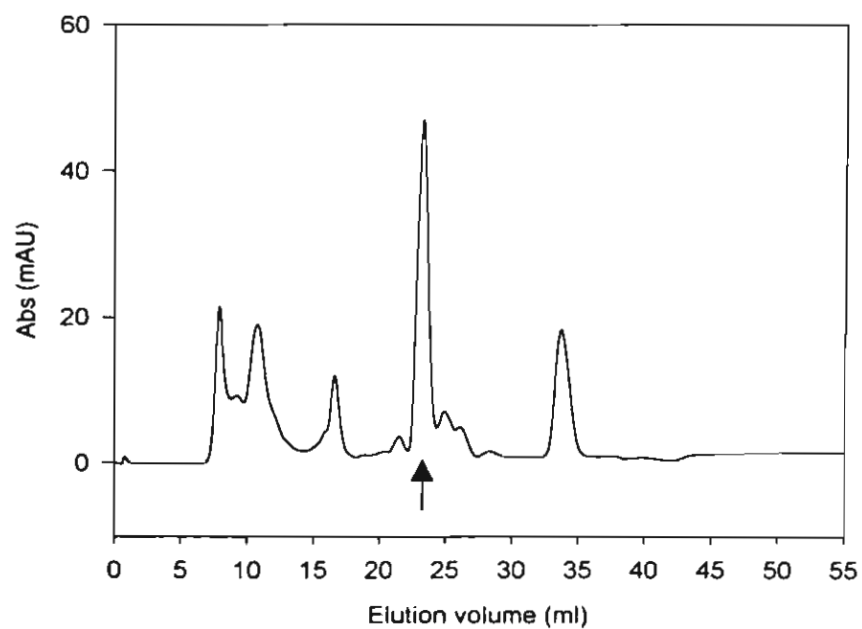


Figure 4: Chromatogram of PEP-2 purification. The purified fraction is labeled with arrow.

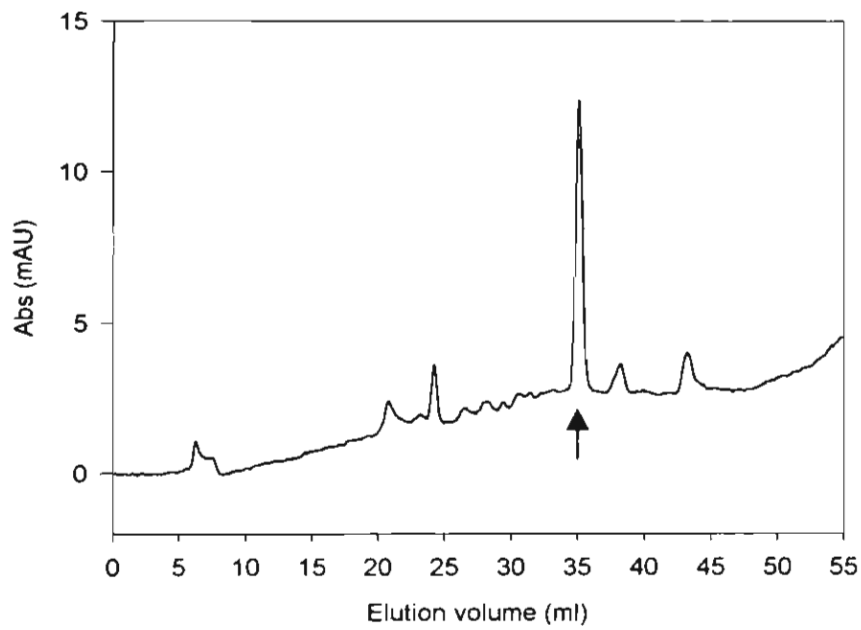


Figure 5: Chromatogram of PEP-3 purification. The purified fraction is labeled with arrow.

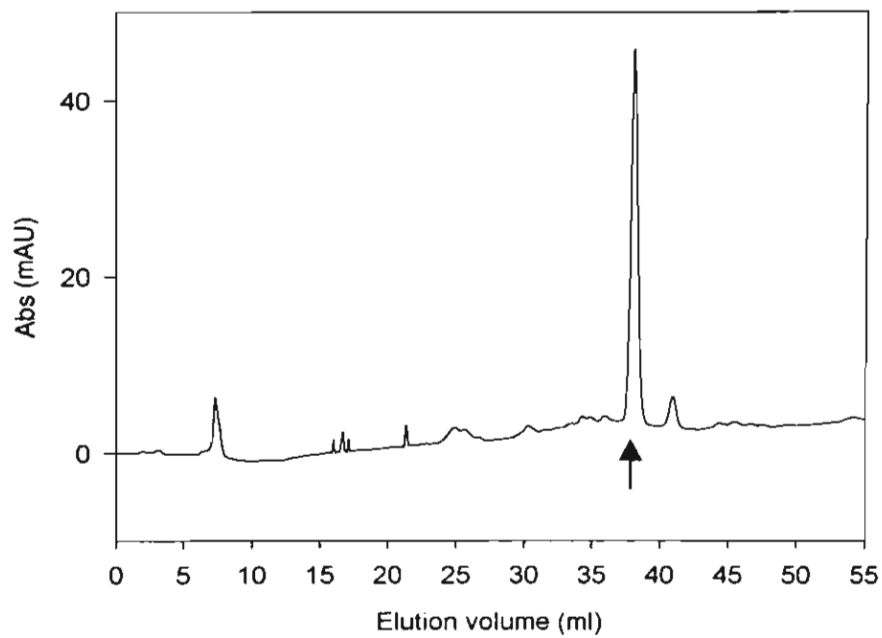


Figure 6: Chromatogram of PEP-4 purification. The purified fraction is labeled with arrow.

Mass Spectrometry

When our purified peptides were loaded and infused into electrospray mass spectrometer, major peaks corresponding to molecular ion of the peptides were detected on the positive ion mode. Their mass to charge ratios (m/z) indicated a singly positive charge on the molecular ion of peptides. Figure 7 demonstrated a typical mass spectrum of the purified peptide showing a single major peak for $[MH]^+$ molecular ion. The m/z at 1677.0 was corresponding to the synthetic PEP-4 peptide of sequence Ac-Y-ALSLAATLLKEAASL-OH. The calculated mass from the amino acid sequence and a capping acetyl group was 1,676 Dalton. It is suggested that the ionization of this peptide give a product containing one extra proton at 1+ charge state for $m/z = [1,676 + 1]^+$ or $[1,677]^+$. Molecular mass analysis for all of our peptides also gave the same pattern of mass spectra and their molecular masses are listed in Table 3.

The molecular mass data from our mass spectroscopy had not only identified the eluted fraction containing peptide of interest from chromatography, but also confirmed that the obtained synthetic peptides were in the correct sequences.

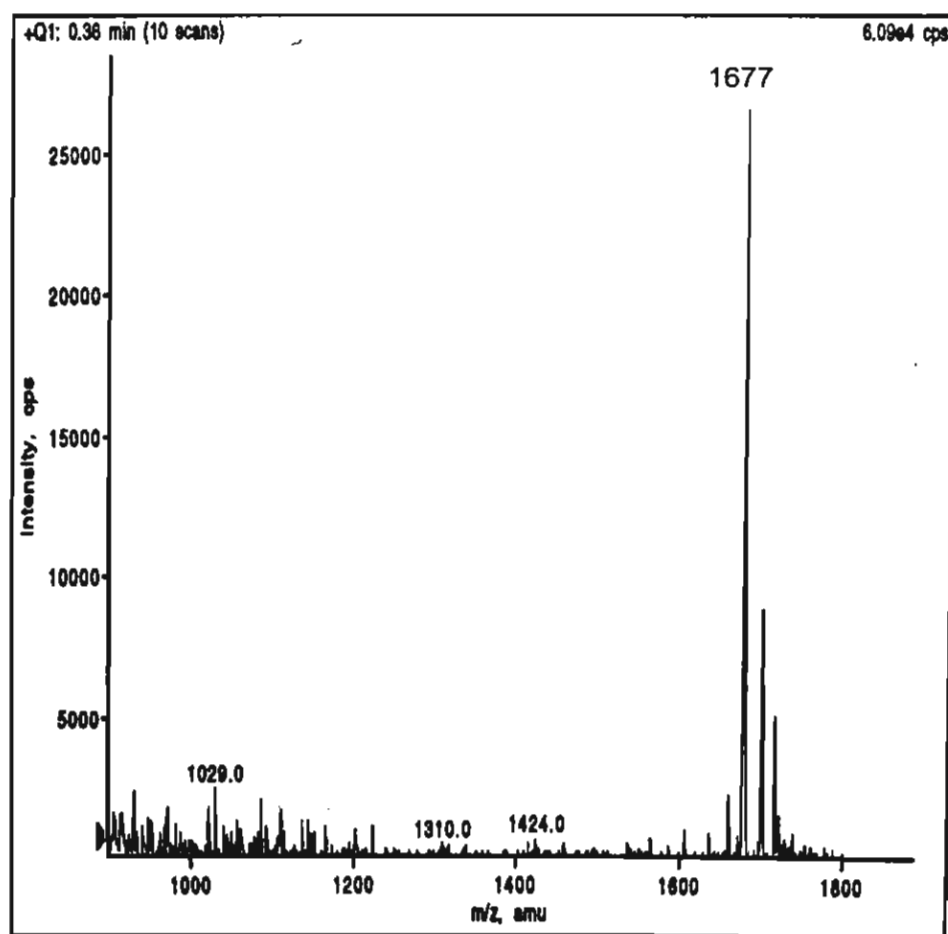


Figure 7: A typical mass spectrum of the purified peptide derived from electrospray mass spectrometer. The intensity is plotted relative to the major mass peak.

Peptides	Sequences	Calculated Molecular Mass	Detected Molecular Mass
PEP-1	Ac-Y-VAAAK-VAAAK-VAAAK-OH	1,543.8	1,545
PEP-2	Ac-Y-ALSEA-KTLLK-EAASL-OH	1,707.9	1,709
PEP-3	Ac-Y-KALAE-ALARA-LADSA-OH	1,633.8	1,635
PEP-4	Ac-Y-ALSLA-ATLLK-EAASL-OH	1,675.9	1,677

Table 3: Molecular masses of synthetic peptides determined from electro-spray mass spectrometry

Determination of Peptide Concentration

All of our designed sequences contain an N-terminal tyrosine (Y) residue to assist in peptide concentration determination. We scanned an absorption band for this tyrosine at 280 nm and use an extinction coefficient of 1,280 for the calculation. The tyrosine absorption band was found to be similar for all of our peptides as shown in Figure 8. In this figure the selected sample was the PEP-4 peptide. The concentration of stock peptides was determined and listed in Table 4.

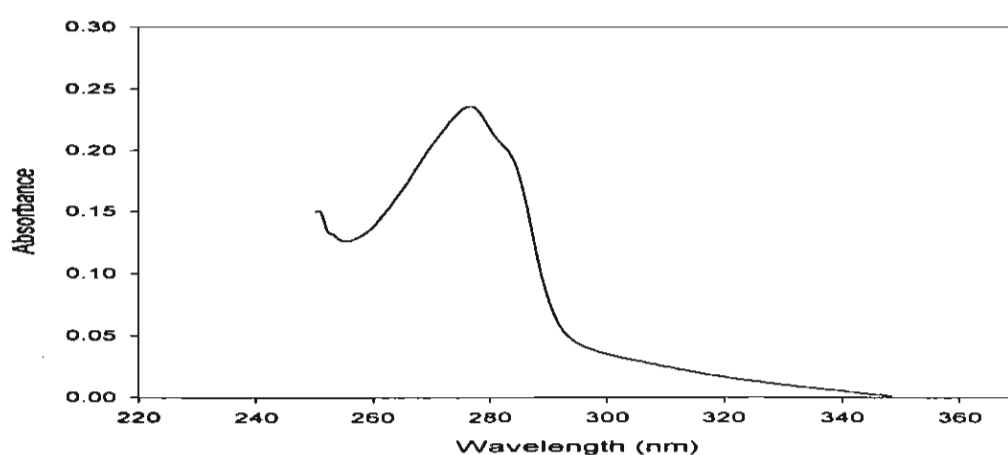


Figure 8: An absorption band of tyrosine around 280 nm used for concentration determination of peptides

Peptides	Sequences	Calculated extinction coefficients	Determined concentration of stock peptide (mM)
PEP-1	Ac-Y-VAAAK-VAAAK-VAAAK-OH	1,280	1.14
PEP-2	Ac-Y-ALSEA-KTLLK-EAASL-OH	1,280	0.96
PEP-3	Ac-Y-KALAE-ALARA-LADSA-OH	1,280	1.32
PEP-4	Ac-Y-ALSLA-ATLLK-EAASL-OH	1,280	1.85

Table 4: Concentration of synthetic peptide stock solutions determined from absorption spectra at 280 nm

Circular Dichroism Spectroscopy

Circular dichroism spectroscopy has been widely recognized as a method of choice for secondary structure analysis. The CD spectra for our purified peptides were measured and analyzed to identify for their structure. Since the secondary conformation of proteins and peptides are known to be solvent dependent (Krittanai and Johnson, 2000), we performed the measurement in both aqueous and organic buffer conditions using phosphate buffer pH 7.2 and 50% CAN respectively.

For aqueous condition all of our peptides gave a typical CD spectra for random conformation. Their spectra contain an intense negative band below 190 nm and negative baseline up from the far-UV to near-UV region (Figure 9, only spectra for PEP-4 are shown). On the other hand, their spectra in an organic solvent were found to resemble a typical alpha helical conformation with an intense positive band for $\pi\pi^*$ around 193 nm and other two negative bands for $n\pi^*$ at 209 and 220 nm. These results were in good agreement with the predicted structures from our molecular modeling. Since this organic buffer are generally used by research to mimic a membrane environment, the formation of alpha helical structure in organic solvent was considered as a good indication for the membrane-associated activity of the peptides.

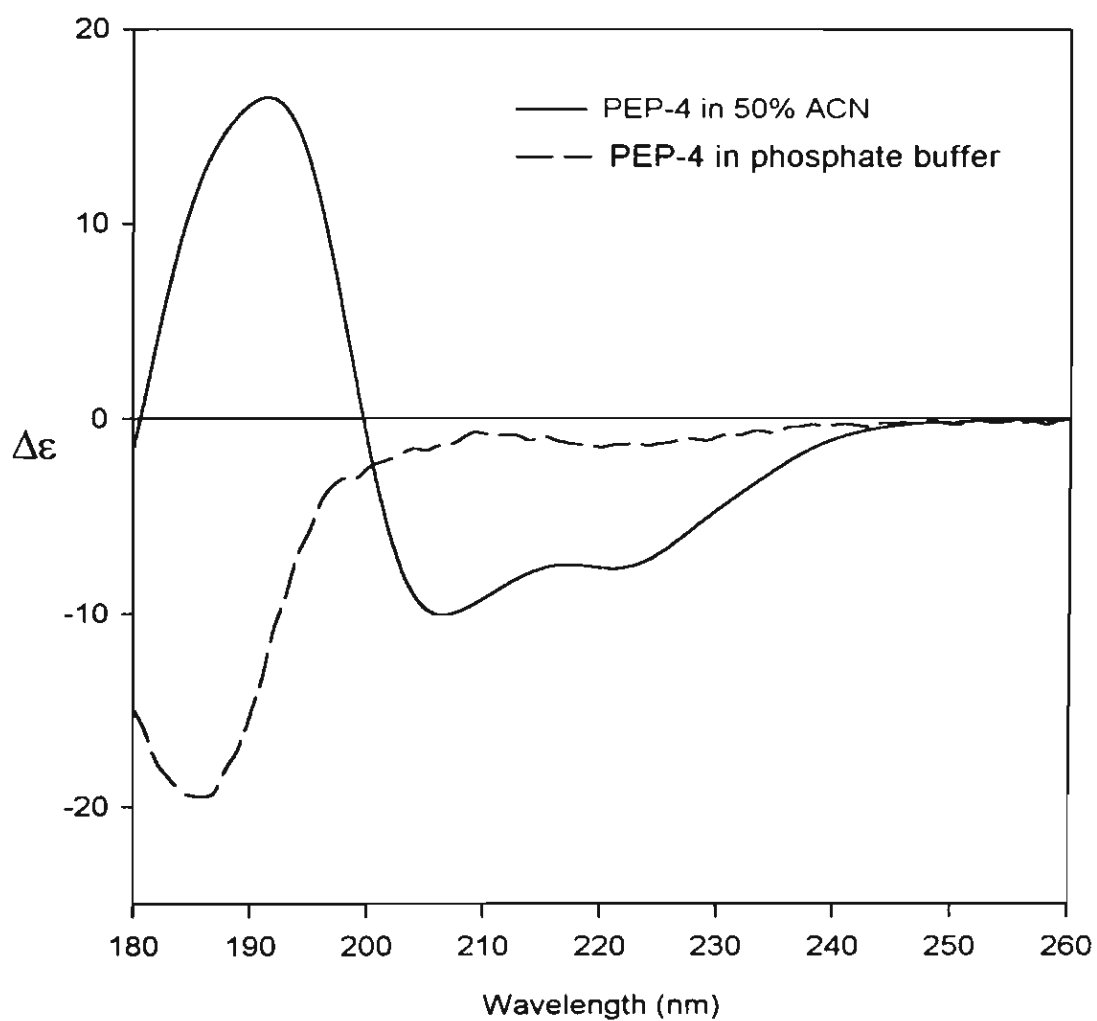
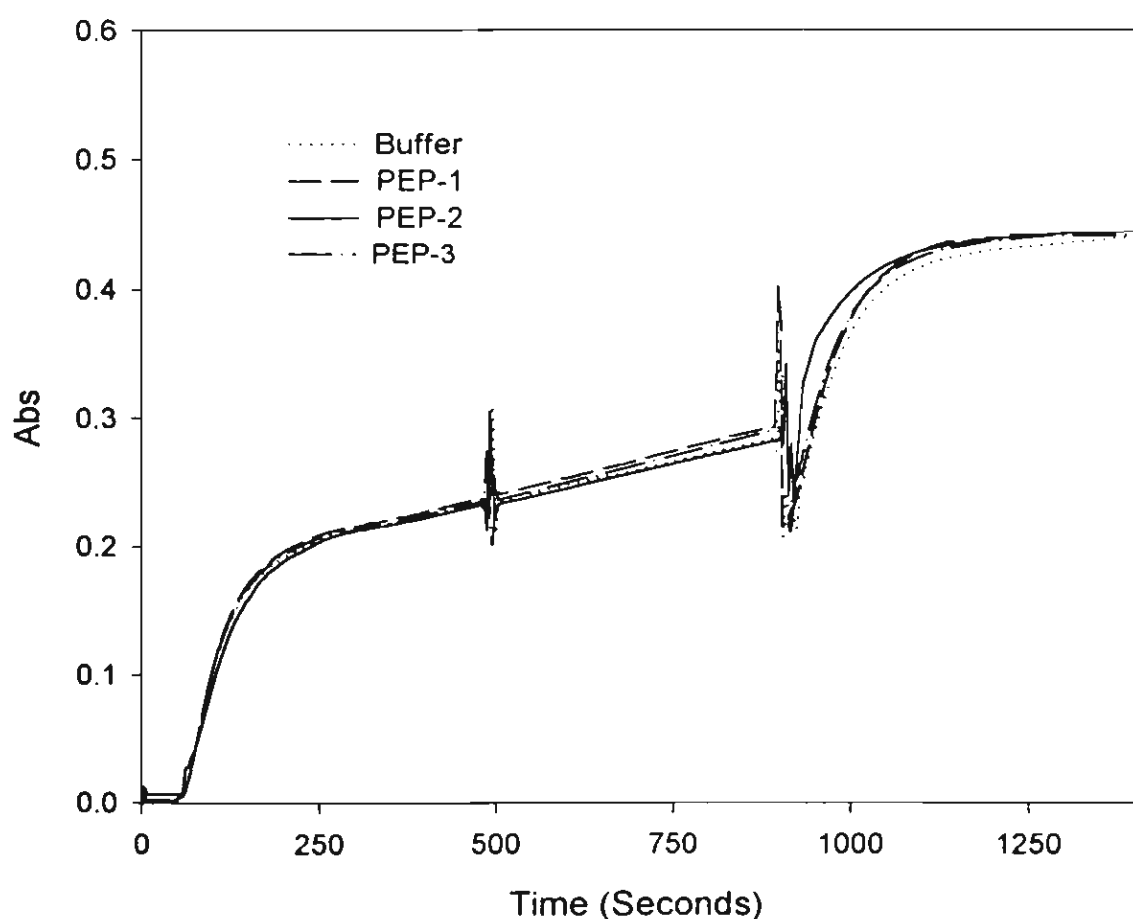


Figure 9: Circular dichroism spectra of peptides in aqueous and organic buffer

Liposome-Entrapped Glucose Release Assay

The small unilamellar vesicle (SUV) was prepared with glucose entrapped inside the vesicle. The vesicle membrane in this work acts as an artificial cell membrane to keep the glucose inside the cell at normal condition. Upon the interaction with the tested peptides, the glucose molecules were monitored outside the vesicle to see whether the vesicle membrane contains a leak or pore induced by the membrane insertion. The measurement of glucose release was based on the conversion of glucose into glucose-6-phosphate (G6P) by hexokinase and then the production of NADPH from NADP by glucose-6-phosphate dehydrogenase activity. The reactions were taken place in the solution surrounding the liposome and their progress was detected by absorption of an increased NADPH. For the experiment using PEP-1, PEP-2, and PEP-3 peptides, we found that an absorption was observed increasing only after step I (where all the enzyme and required substrates were mixed into the liposomes) and step III (where the tritonX-100 was added to break all the liposomes for a total release). From the measurement (Figure 10) we did not observe any significant increased absorption in step II that the peptide was added in to the system. This is suggesting that the peptides PEP-1, PEP-2, and PEP-3 were not capable of releasing glucose



from the liposome vesicle.

Figure 10: Changes of absorption resulting from glucose release experiment using PEP-1, PEP-2 and PEP-3 peptides. Step I, II, and III are the times of adding enzyme-substrate cocktail, peptide, and triton-X respectively.

However the experiment has showed a glucose release activity from PEP-4 peptide. The absorption was found to have a marked increase upon the adding for this peptide in step II (Figure 11). We repeated the experiment by using different concentration at 10 and 20 μM of the PEP-4 peptide and found difference in the activity. This result has indicated that our PEP-4 peptide can interact with the liposome and cause the membrane leakage as shown by the detection of glucose outside the vesicle.

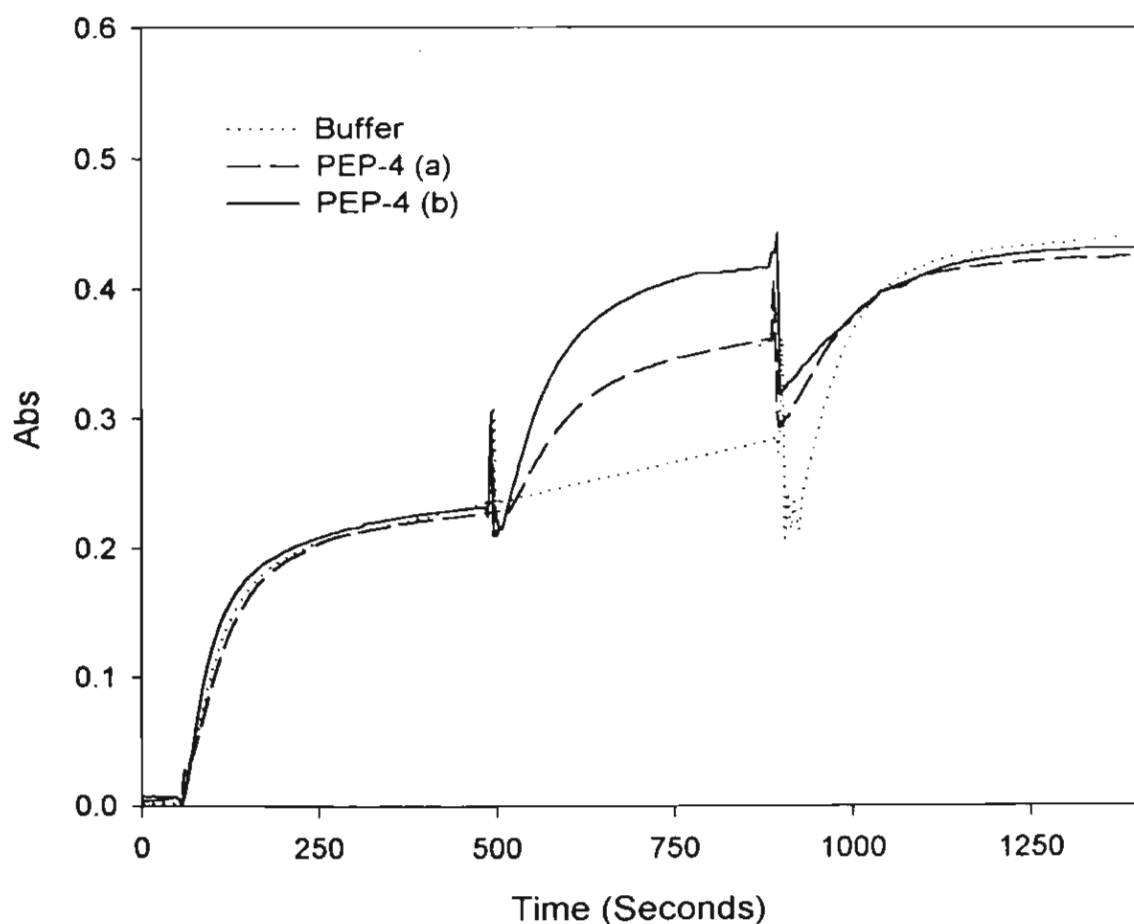


Figure 11: Changes of absorption resulting from glucose release experiment using PEP-4 peptide. Step I, II, and III are the times of adding enzyme-substrate cocktail, peptide, and triton-X respectively. (a) and (b) are marked for peptide concentration of 10 and 20 μM .

Ion Channel Activity Assay by Planar Lipid Bilayer System

A detection of glucose-release activity from the liposome assay has suggested that our PEP-4 peptide is capable of forming a pore on the unilamellar lipid vesicle. A further investigation for a pore-forming activity of this peptide was performed on a planar lipid bilayer (PLB) system. Even through the setup and sample preparation for PLB experiment is complicated, but the experimental results can give a number of qualitative and quantitative data enough to describe for the ion channel property of the peptide. In our PLB experiment, we performed both symmetrical and asymmetrical assays for the purified PEP-4 peptide. While the results from a symmetrical system can be used to determine a conductance value that is specific for each ion channels, asymmetrical system can reveal whether the channels are cation or anion selective.

A general PLB system contains two solution chambers, *cis* and *trans*, connected to each other through a small aperture painted with a lipid film. Our symmetrical system was filled with 150 mM KCl in both chambers, while the asymmetrical system was filled with 450 and 150 mM KCl in *cis* and *trans* chambers respectively. The command potentials were applied through small electrodes located inside the two chambers and electrical current was monitored for various potentials.

Symmetrical planar lipid bilayer experiments

Planar lipid bilayer experiment in symmetrical mode was performed to deduce the channel conductance. This parameter represents a characteristic value of the pore on lipid membrane. The results were obtained and depicted as a plot between the current observed versus progressive time period (Figure 12 to Figure 20). Our observation period for baseline experiment at 0 millivolt of applied potential was set 30-45 minutes to test for lipid membrane stability. Data derived from experiments with applied potentials from -100 to +100 millivolts were collected for 60,000 to 100,000 milliseconds on a computer and only 2,000 milliseconds were shown in the figures.

Ion channel assay at 0 millivolt

At the starting condition, a command potential of 0 millivolts was applied to the electrodes and the current signal was monitored against time for more than 45 minutes. The current was detected at the zero value as an indication for no channel activity. This condition was then used as a reference or baseline condition for other experiments.

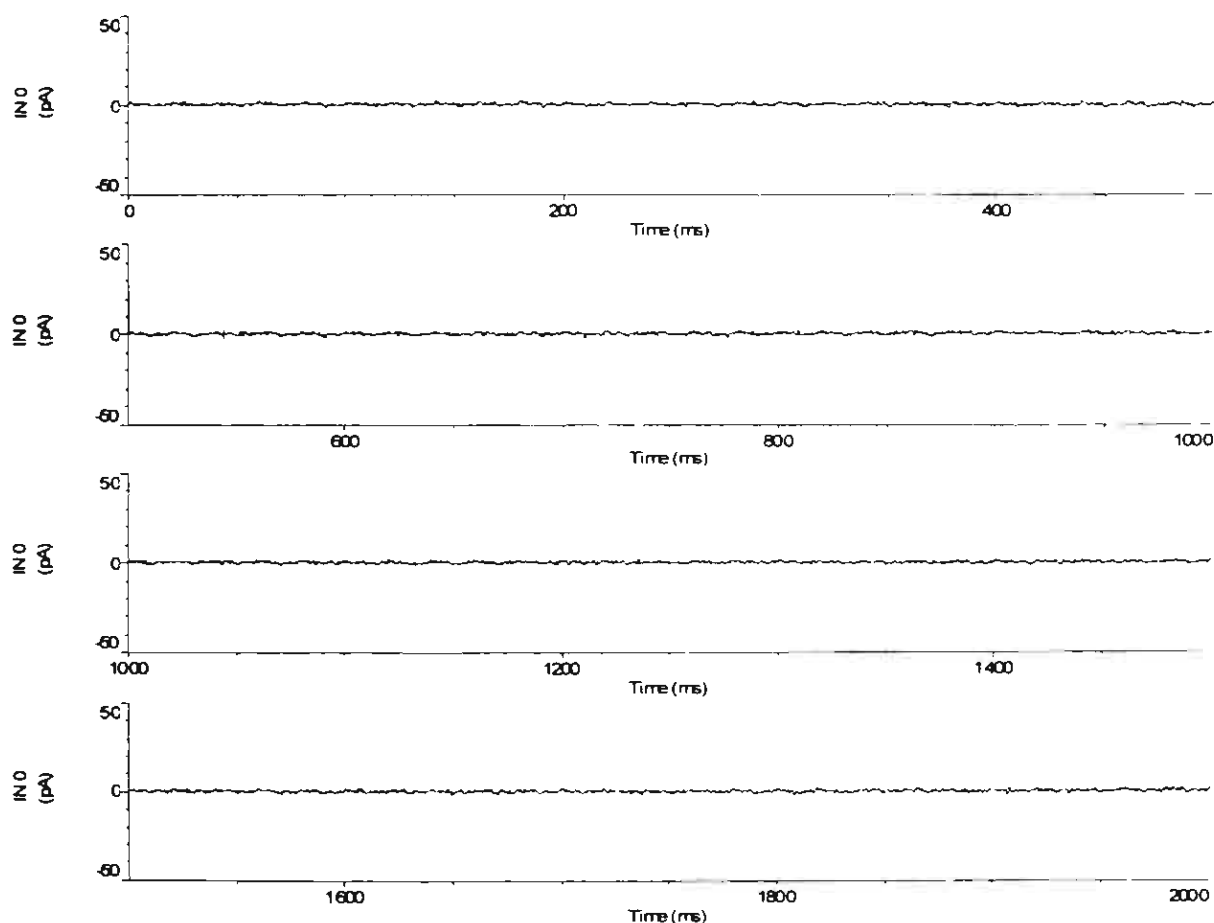


Figure 12: A plot between current and time obtained from planar lipid bilayer experiment at applied command potential of 0 millivolt. The time period shown is from 0 to 2000 milliseconds.

Ion channel assay at -100 millivolts

When a command potential of -100 millivolt was applied to the, we found a current jump from zero to a negative current signal. Signal analysis by AXOFit software gave a current value of -22.2 picoamperes. It is suggested that the channel activity were established on the membrane allowing ions to pass across and generate electrical current. The stability of the current indicated that the channel was in open state at all times.

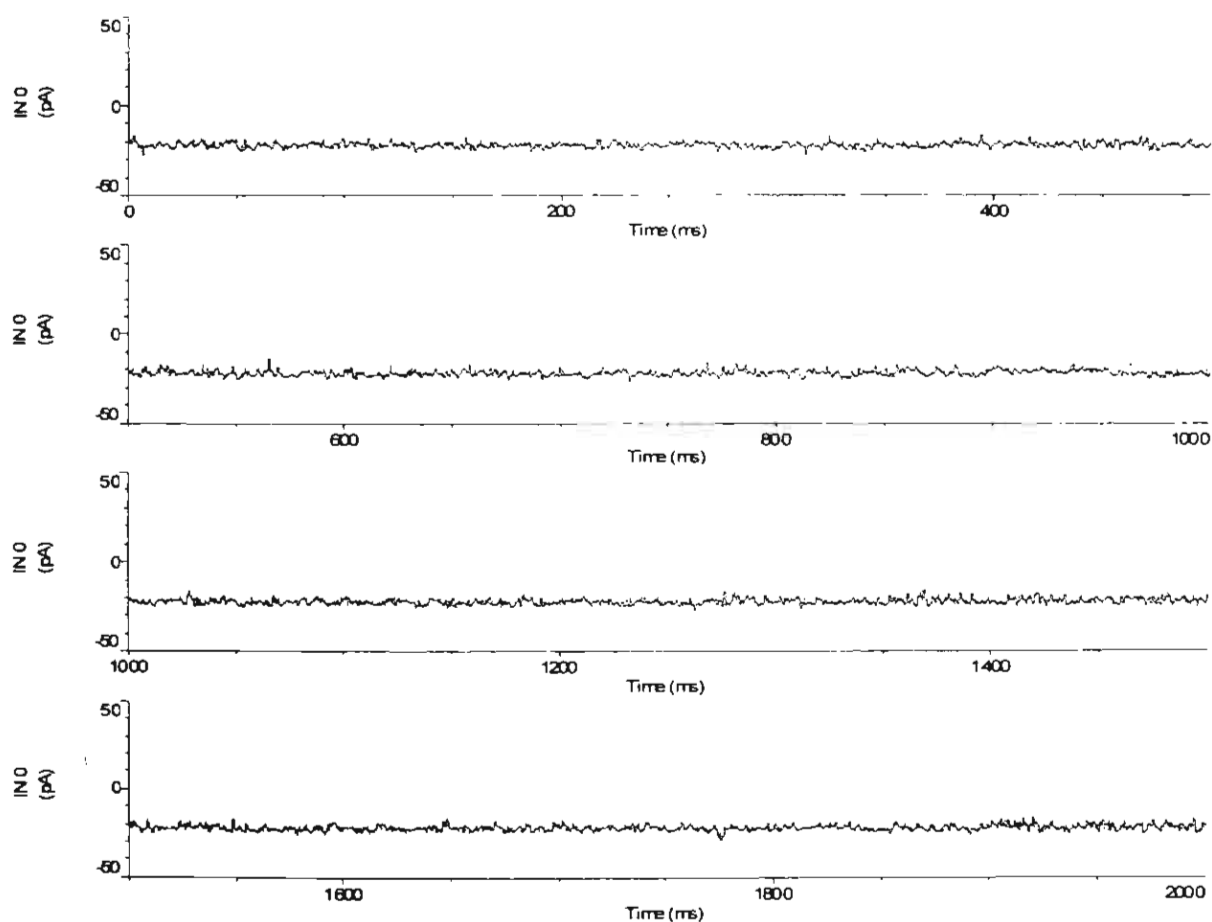


Figure 13: A plot between current and time obtained from planar lipid bilayer experiment at applied command potential of -100 millivolts. The time period shown is from 0 to 2000 milliseconds.

Ion channel assay at +100 millivolts

When a command potential of +100 millivolts was applied to the electrodes, we found a current jump from a negative to a positive current signal. Signal analysis by AXOFit software gave a current value of +18.1 picoamperes. It was suggested that there was an ion channel activity on the membrane. The current signal was found with a numbers of square jumps down to zero current indicating a transition from an opened to a closed state. However the open states were predominant than the closed state.

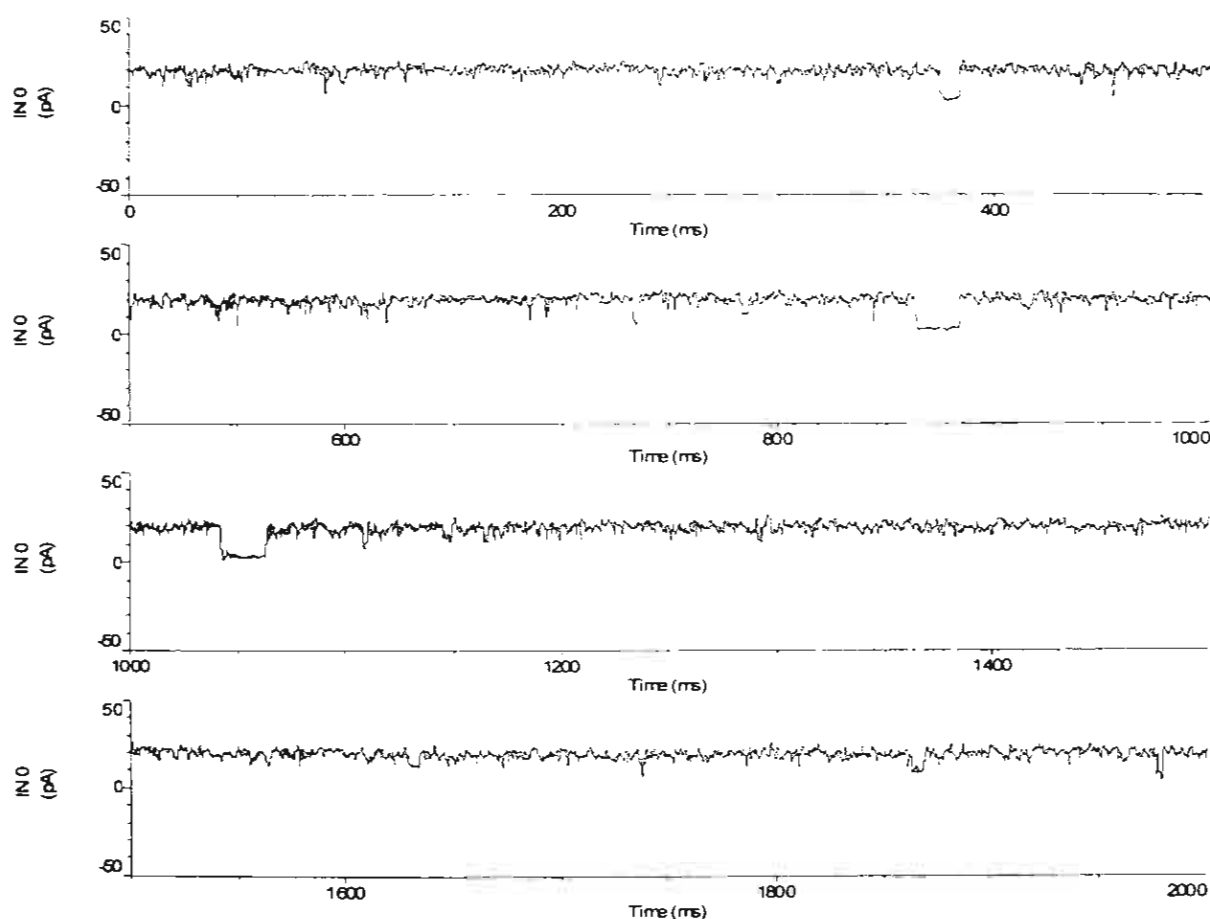


Figure 14: A plot between current and time obtained from planar lipid bilayer experiment at applied command potential of +100 millivolts. The time period shown is from 0 to 2000 milliseconds.

Ion channel assay at -80 millivolts

When a command potential of -80 millivolts was applied to the electrodes, we found a current jump to a negative current signal. Signal analysis gave a current value of -14.2 picoamperes. This data suggested a channel activity on the membrane. The current signal was stable on the negative mode indicating an open state of channel.

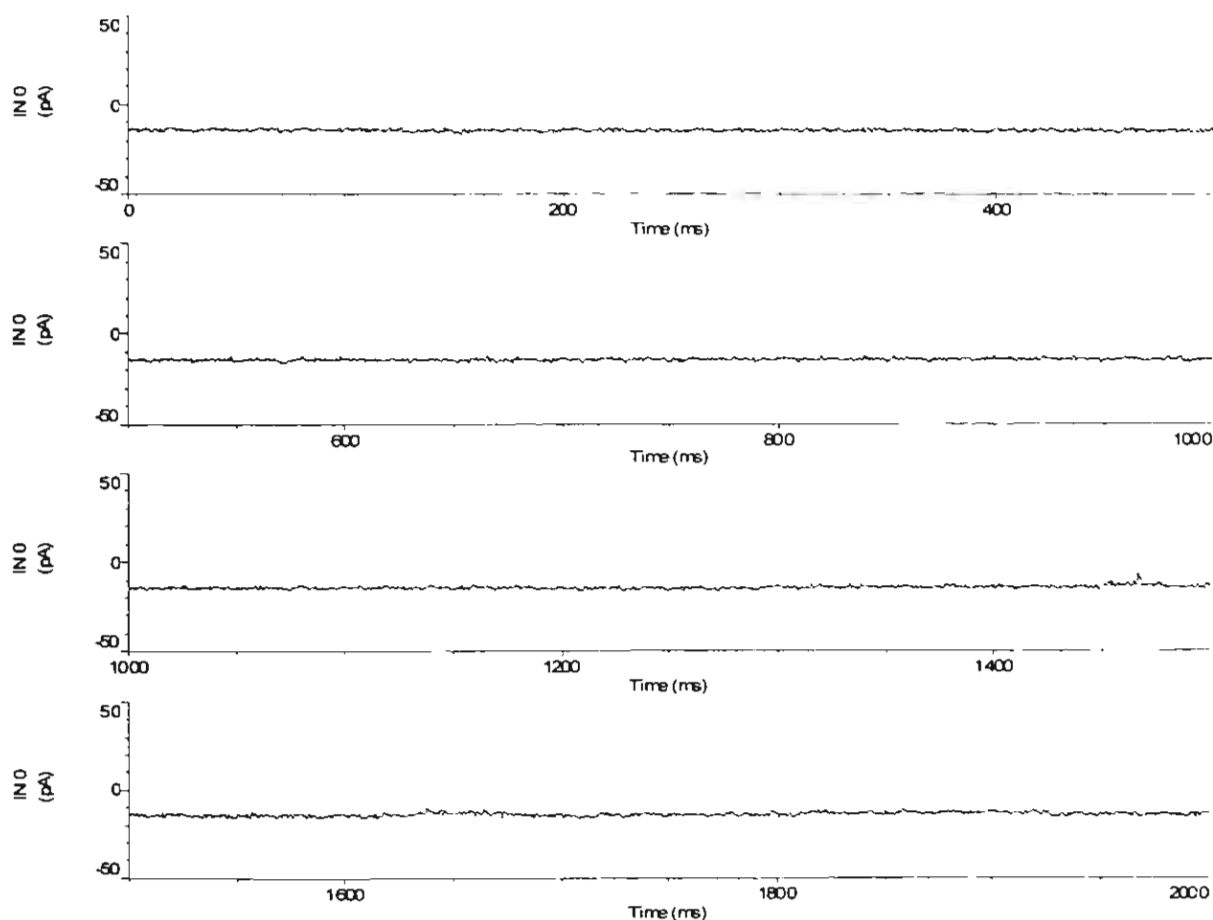


Figure 15: A plot between current and time obtained from planar lipid bilayer experiment at applied command potential of -80 millivolts. The time period shown is from 0 to 2000 milliseconds.

Ion channel assay at +80 millivolts

When a command potential of -80 millivolts was applied to the electrodes, we found a current jump to a positive current signal. Signal analysis gave a current value of +11.3 picoamperes. This data demonstrated a channel activity on the membrane. The current signal was found to have square jumps back to zero current frequently showing a defined transition between open and close states of the channels

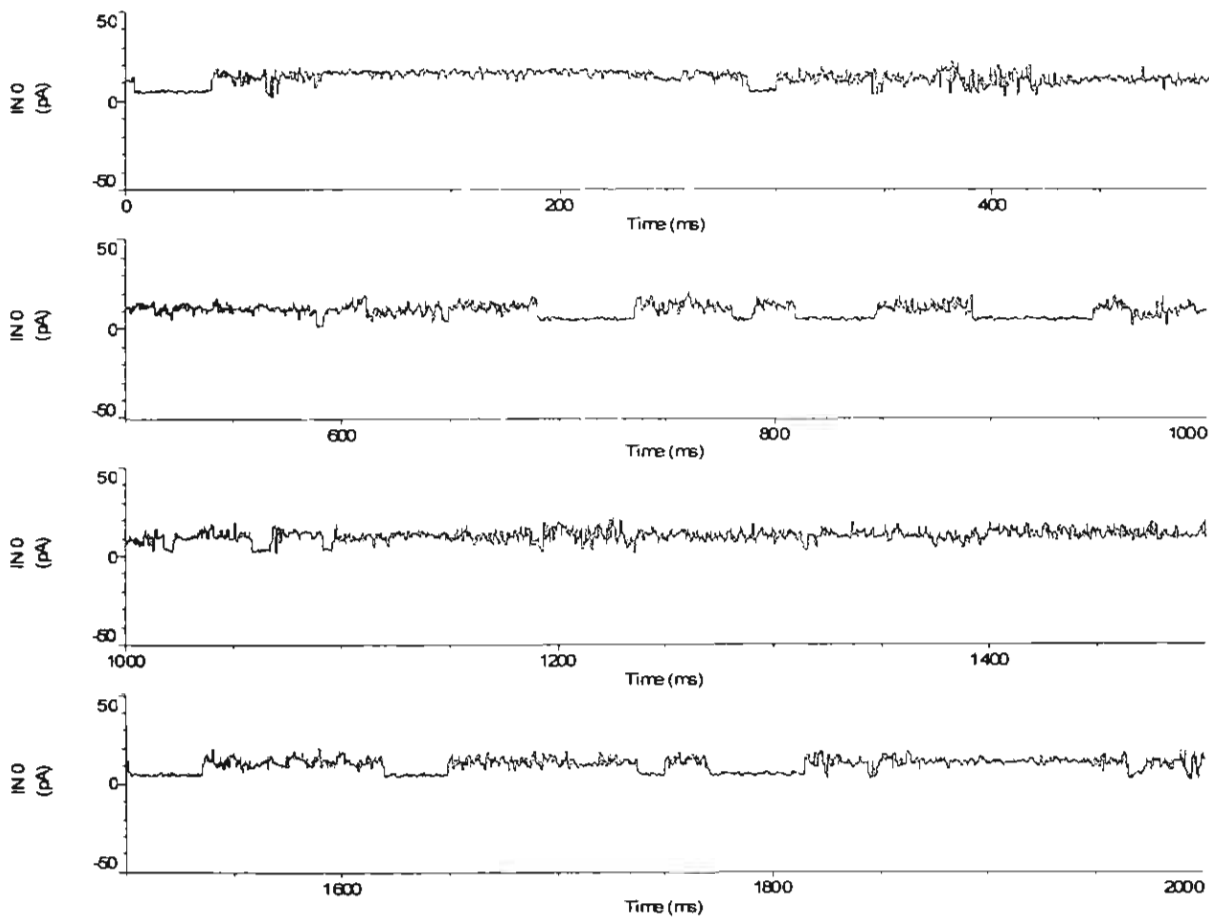


Figure 16: A plot between current and time obtained from planar lipid bilayer experiment at applied command potential of +80 millivolts. The time period shown is from 0 to 2000 milliseconds.

Ion channel assay at -60 millivolts

After a command potential of -60 millivolts was applied to the system, we found a current jump to a negative current signal. Signal analysis gave a current value of -12.8 picoamperes. It was an indication for the channel activity on the membrane. The current signal was found with no square jumps back to the zero current revealing that the channels were in the open state.

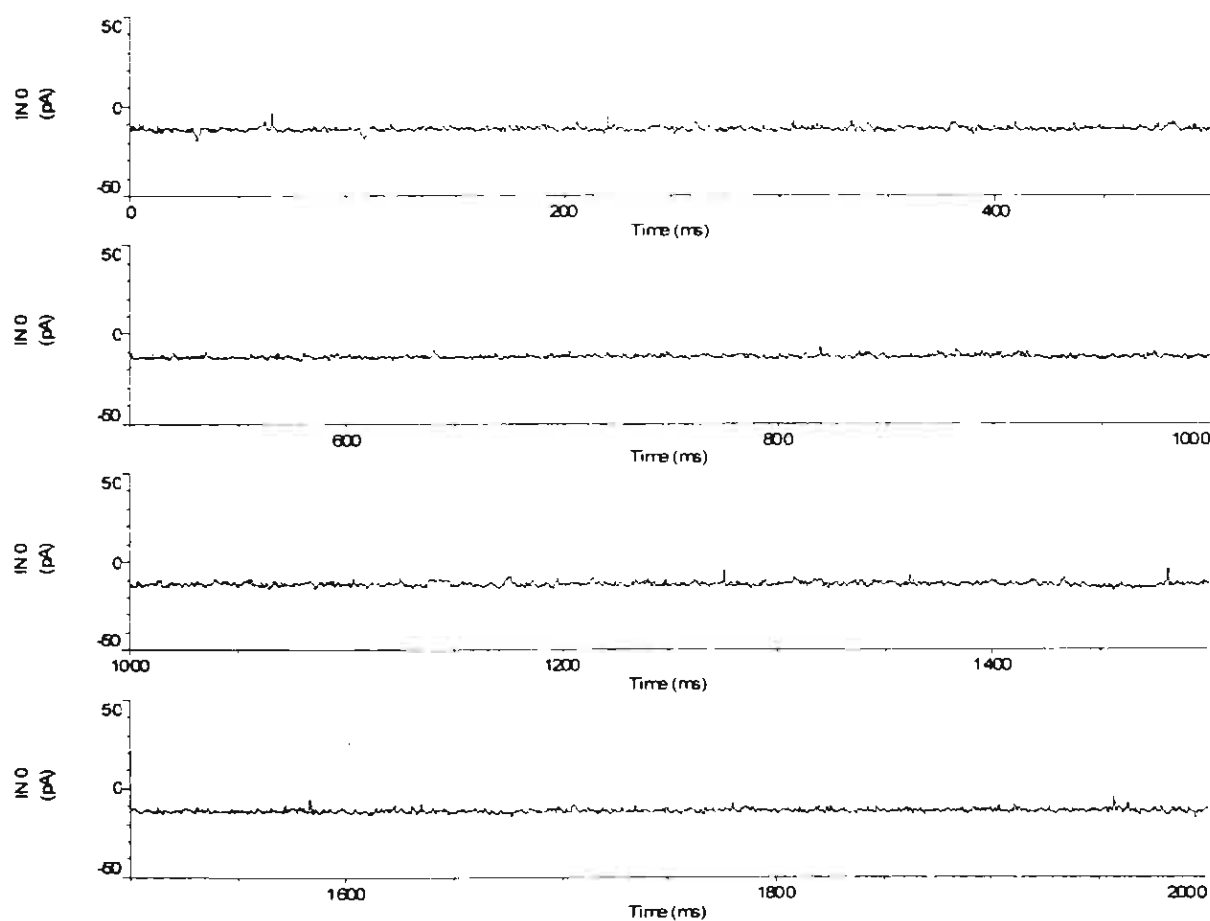


Figure 17: A plot between current and time obtained from planar lipid bilayer experiment at applied command potential of -60 millivolts. The time period shown is from 0 to 2000 milliseconds.

Ion channel assay at +60 millivolts

When a command potential of +60 millivolts was applied to the system, we found a current jump to a positive signal. Signal analysis yielded a current value of +11.0 picoamperes. It was an indicator for the channel activity on the membrane. The current signal was found to have no square jumps back to zero current revealing that the channel was in an open state.

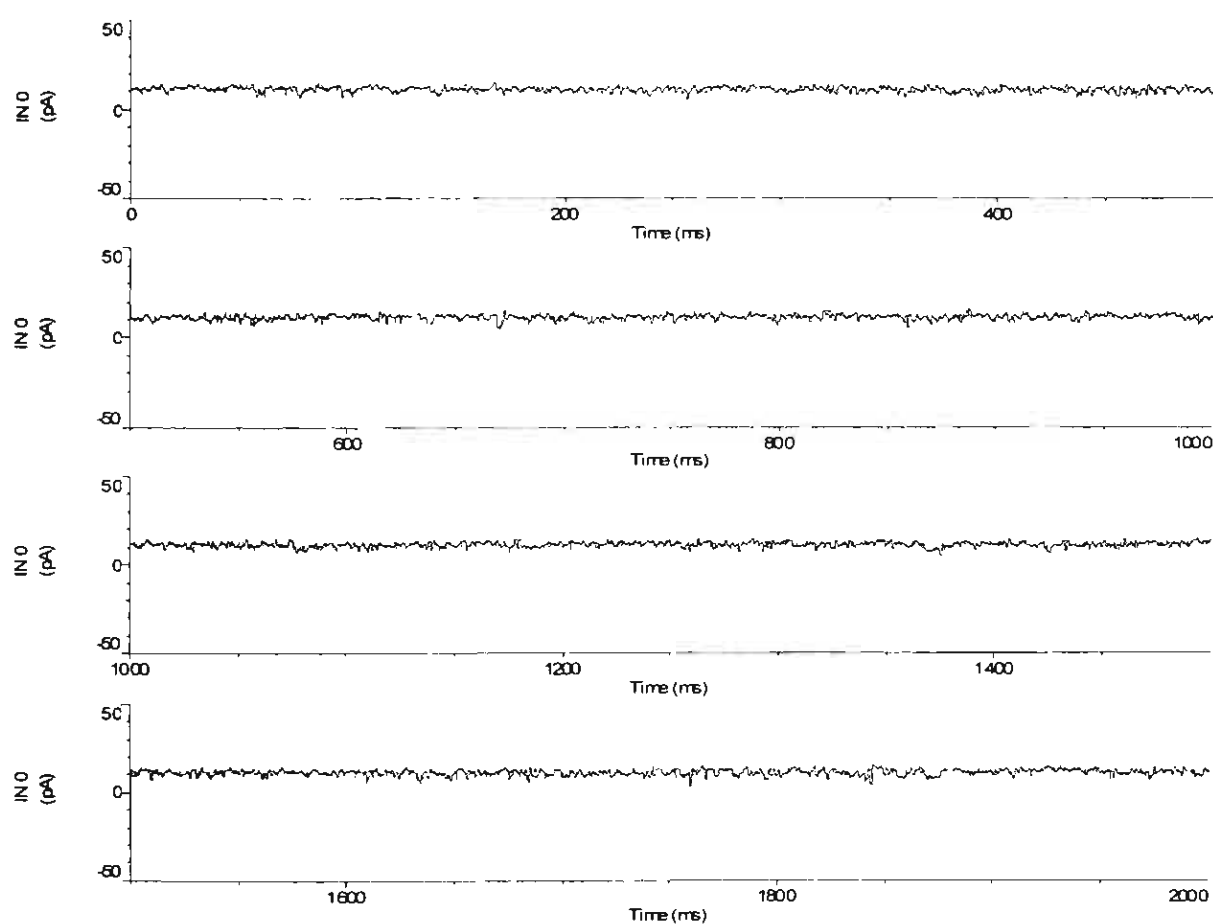


Figure 18: A plot between current and time obtained from planar lipid bilayer experiment at applied command potential of +60 millivolts. The time period shown is from 0 to 2000 milliseconds.

Ion channel assay at -40 millivolts

A command potential of -40 millivolts applied to the system had led to a current jump to a negative signal. Signal analysis gave a current value of -8.4 picoamperes. It was an indication for the channel activity on the membrane. The current signal was found with a few square jumps back to the zero current showing a transition from open to close state. However the open state were much more predominated than the closed state.

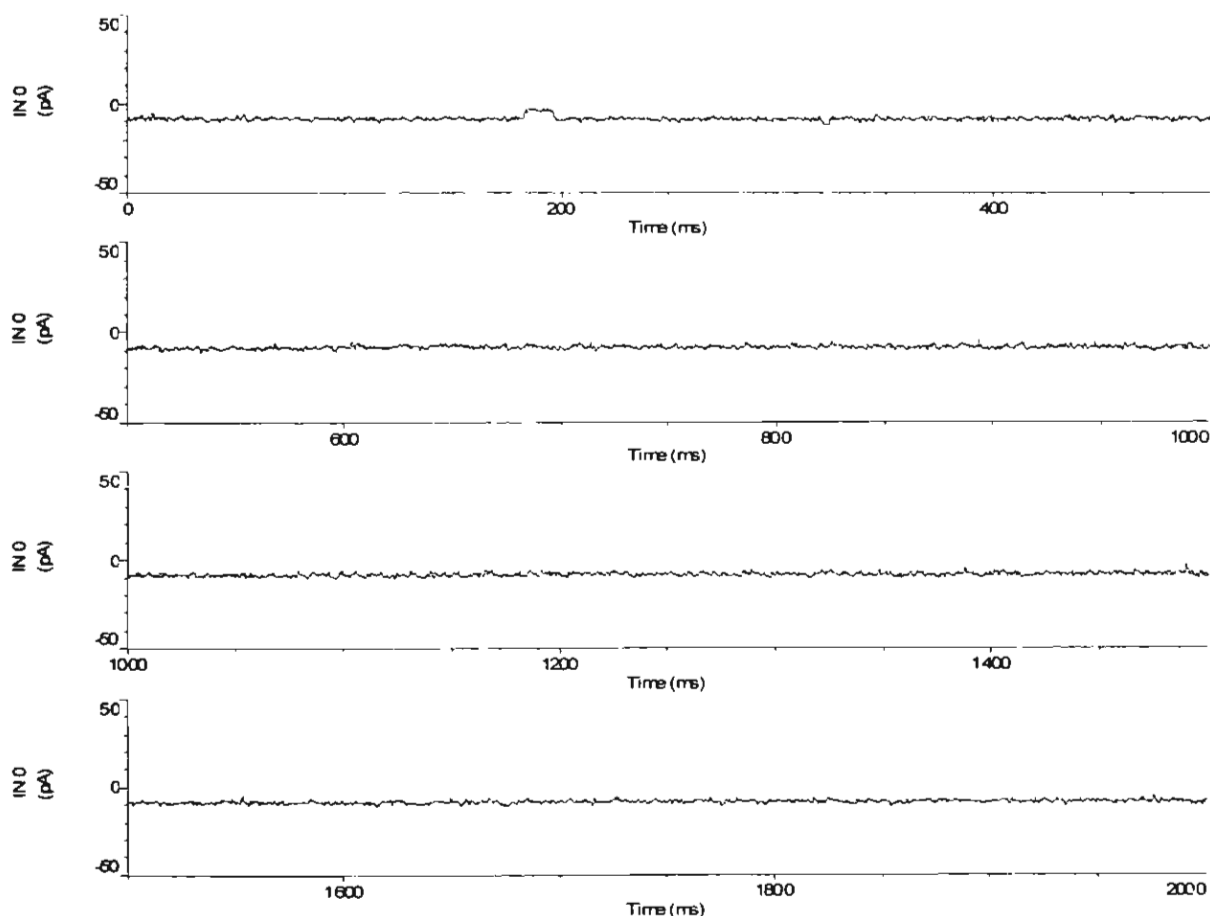


Figure 19: A plot between current and time obtained from planar lipid bilayer experiment at applied command potential of -40 millivolts. The time period shown is from 0 to 2000 milliseconds.

Ion channel assay at +40 millivolts

After a command potential of +40 millivolts was applied to the system, we found a current jump to a negative current signal. Signal analysis yielded a current value of +7.0 picoamperes. It was an indication for the channel activity on the membrane. The current signal was found with no square jumps back to the zero showing open state of channel.

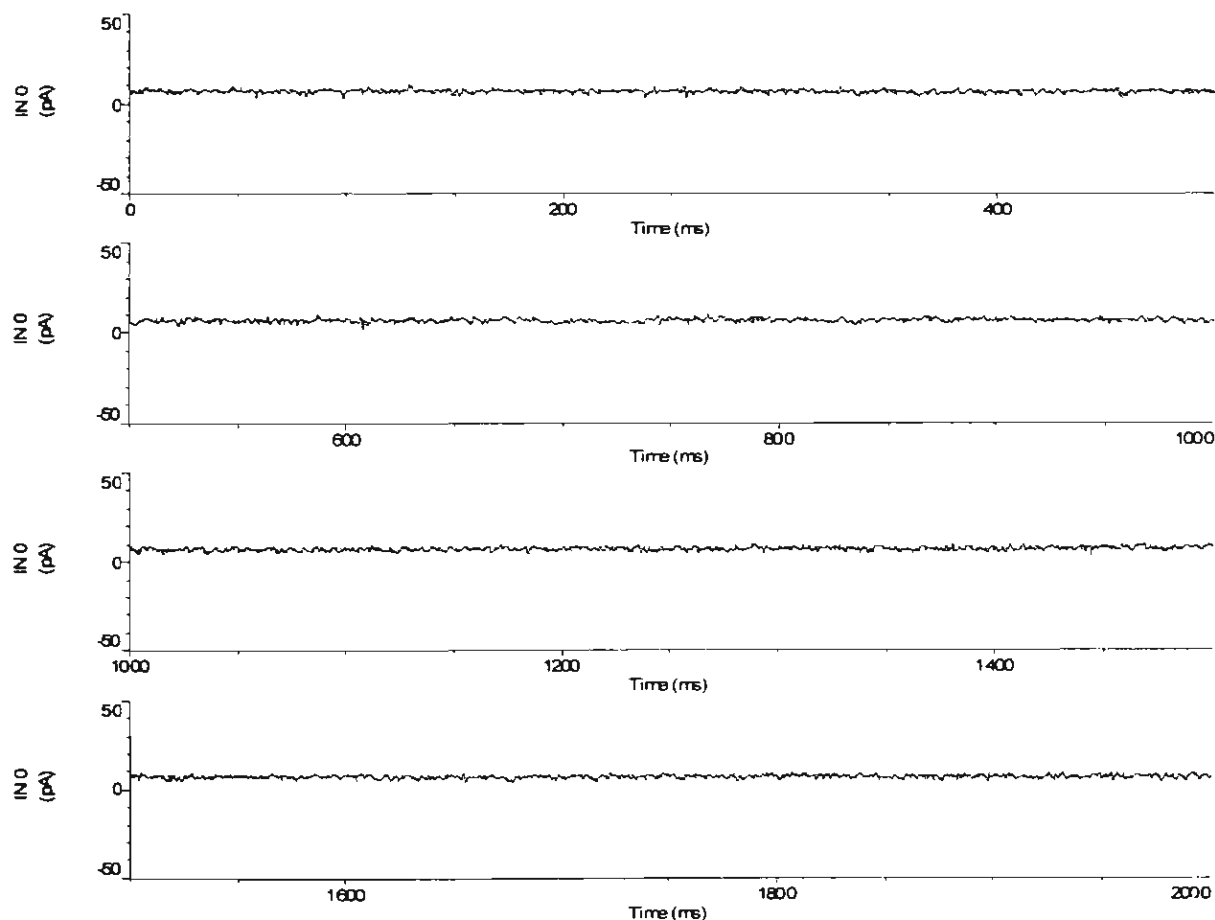


Figure 20: A plot between current and time obtained from planar lipid bilayer experiment at applied command potential of +40 millivolts. The time period shown is from 0 to 2000 milliseconds.

Based upon the zero current detected for the baseline at 0 millivolt potential, the nonzero current detected for all cases with applied potentials from -100, -80, -60, -40, +40, +60, +80, +100 millivolts indicated an ion channel activity of the PEP-4 peptide. The sign of detected currents were found to be corresponding to the sign of applied potentials. We found several square jumps of current to zero ampere and back to their stable values in several conditions (for +100, +80, and +60 mV potentials). These current jumps represent a rapid transition between an open state (nonzero current) and close state (zero current). However the channel observed in our experiments were found to spend most of their time in an open state.

The PLB experiment in symmetrical mode has allowed us to determine a conductance for this ion channel via a plot of detected currents against applied potentials. According to Ohm's law a slope of this plot (I/V) is a reciprocal of resistance or defined as a conductance.

$$I / V = 1 / R = \text{Conductance}$$

The coordinates for the I and V plot was listed in table 5, corresponding to detected currents in picoamperes and the applied potentials in millivolts.

Applied Potentials (mV)	Detected Currents (pA)
0	0.0
- 100	- 22.2
+ 100	+ 18.1
- 80	- 14.2
+ 80	+ 11.3
- 60	- 12.8
+ 60	+ 11.0
- 40	- 8.4
+ 40	+ 7.0

Table 5: Detected current values at various applied potentials obtained from symetric planar lipid bilayer experiment.

A plot of I against V has produced a linear relationship with correlation factor (R^2) of 0.988. The slope determined from the fitted equation, $I = 0.188V - 1.133$ was 188 pS. This means that the ion channel produced from PEP-4 peptide has a characteristic conductance of 188 pS.

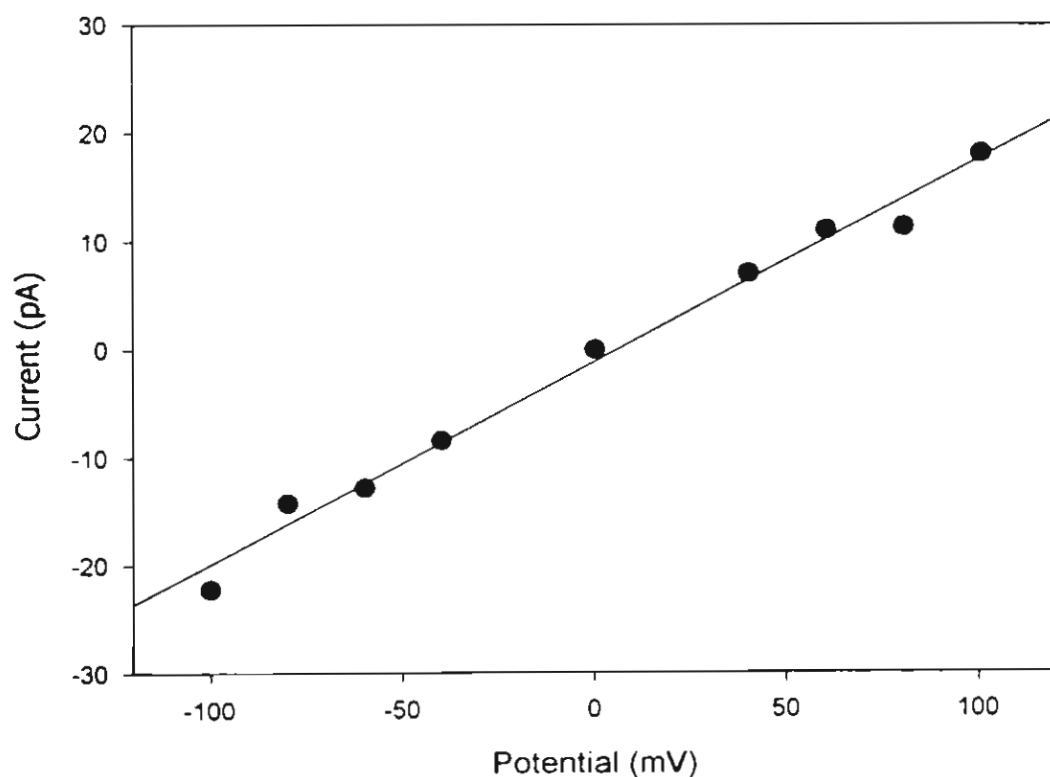


Figure 21: A plot between applied potentials and detected currents obtained from symmetrical planar lipid bilayer experiment.

Asymmetrical planar lipid bilayer experiments

We continued the experiment on planar lipid bilayer in an asymmetrical mode aiming to determine the type of ion channel. *Cis* chambers was filled with a higher concentration of KCl (450 mM against 150 mM) and the modulated activity was monitored at various potentials. The data were depicted as a plot between detected currents and progressive time periods (Figure 22 to Figure 29)

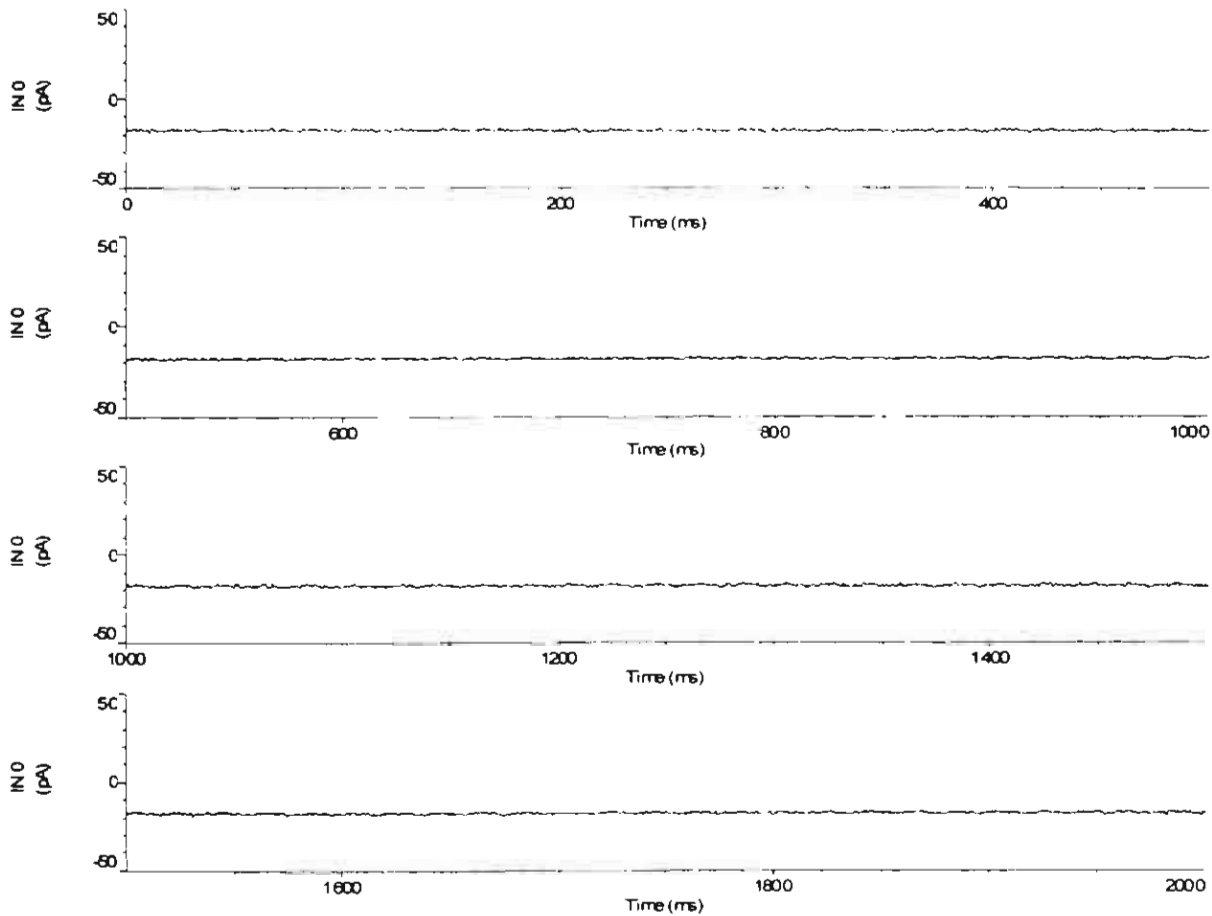


Figure 22: A plot between current and time for asymetric planar lipid bilayer experiment at applied potential -80 millivolts. Signal analysis gave the current value at -18.5 picoamperes.

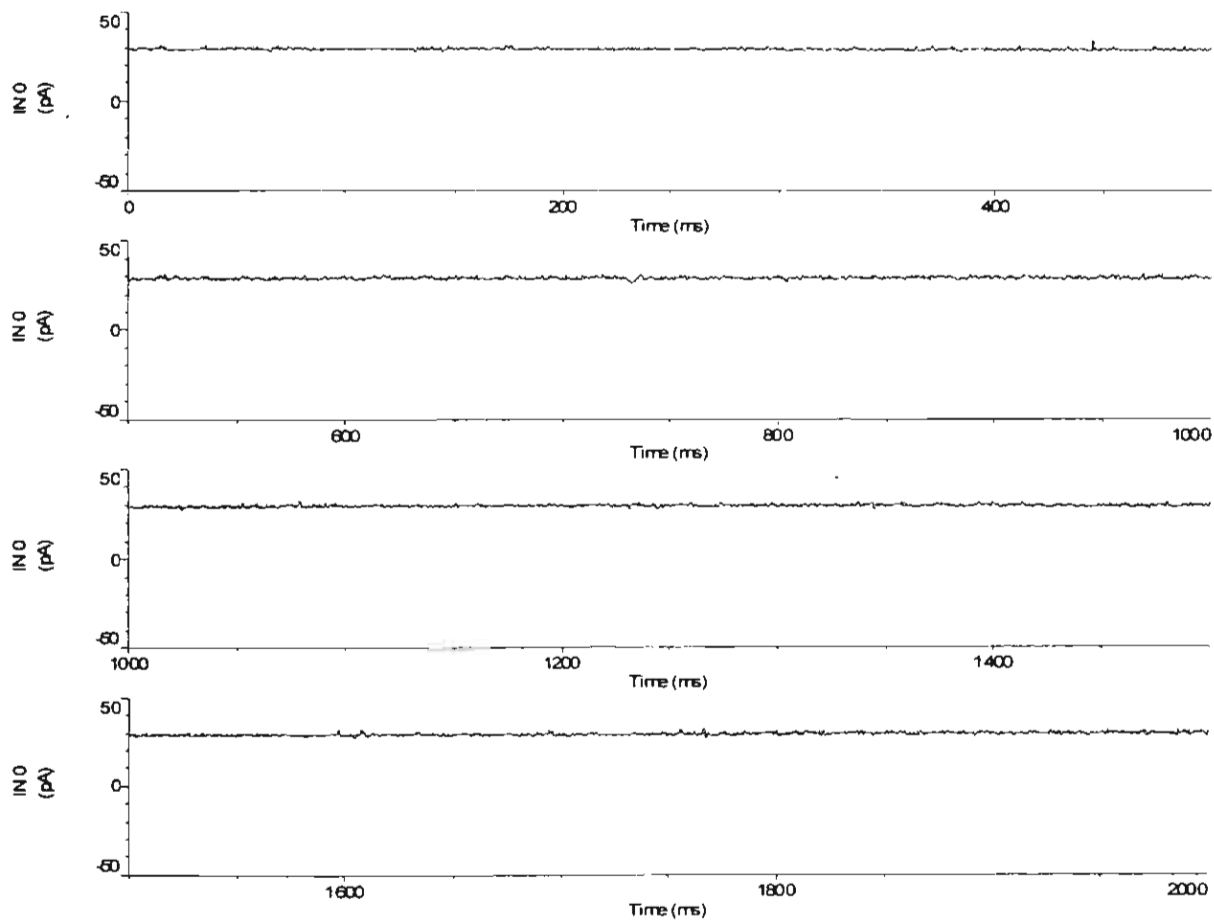


Figure 23: A plot between current and time for asymmetric planar lipid bilayer experiment at applied potential +80 millivolts. Signal analysis gave the current value at +29.0 picoamperes.

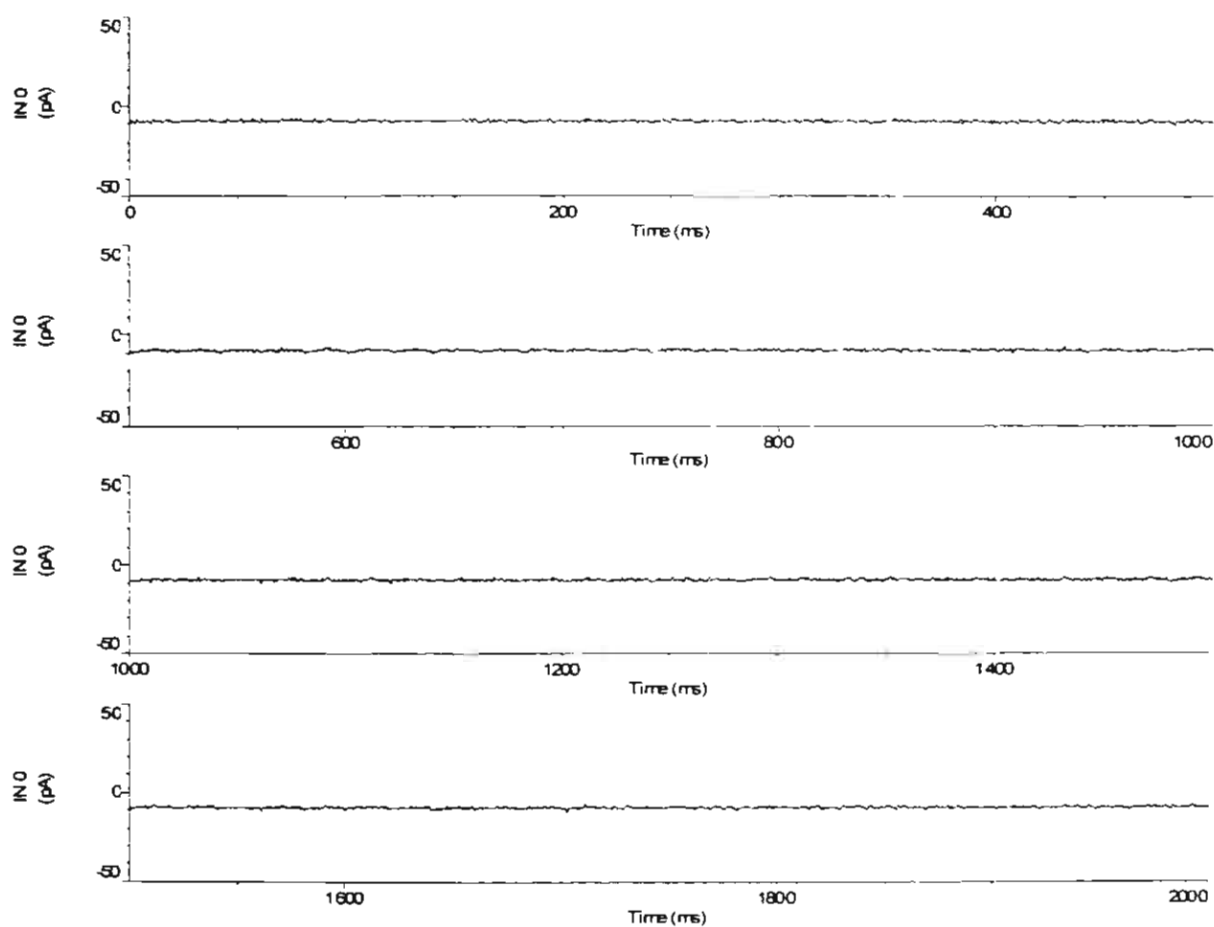


Figure 24: A plot between current and time for asymmetric planar lipid bilayer experiment at applied potential -50 millivolts. Signal analysis gave the current value at -8.4 picoamperes.

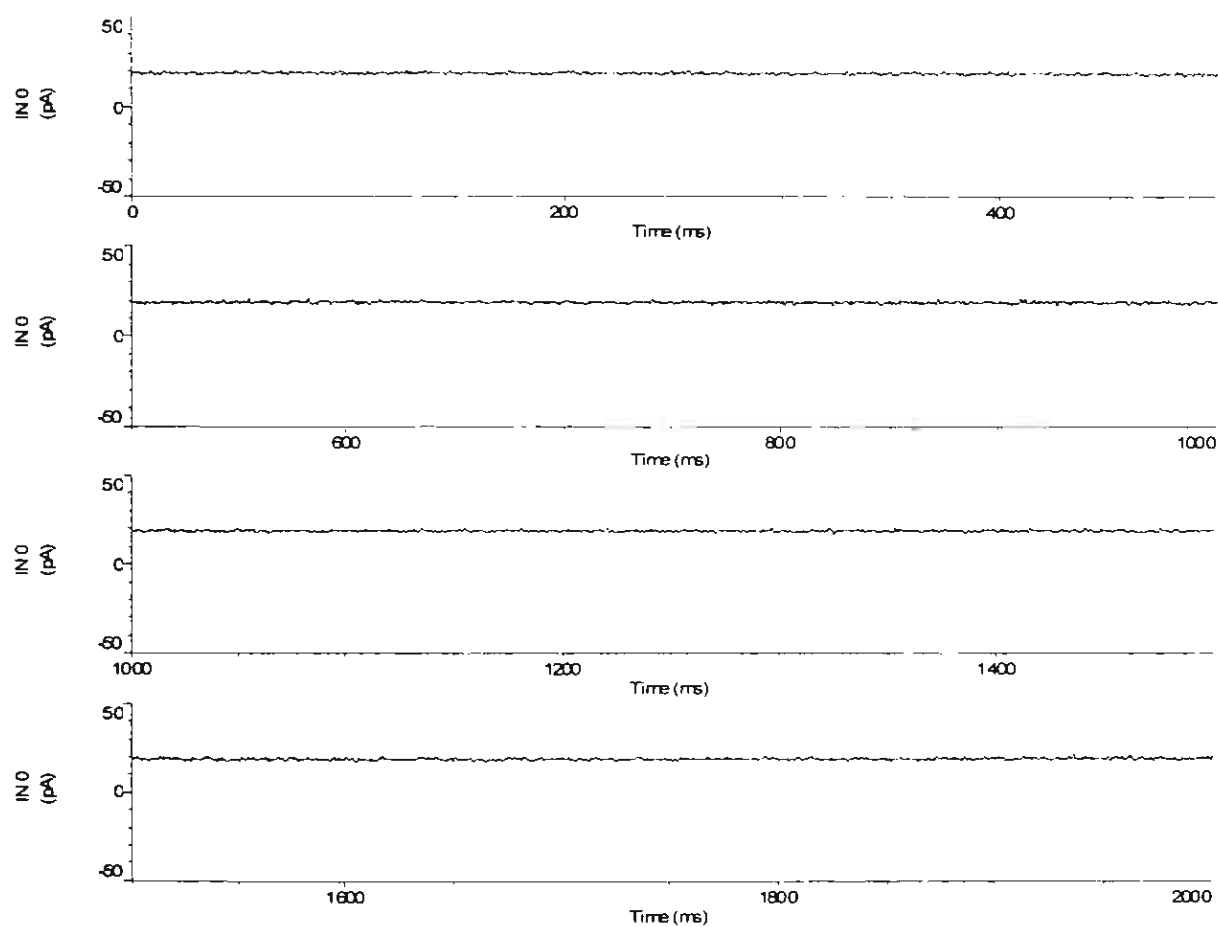


Figure 25: A plot between current and time for asymmetric planar lipid bilayer experiment at applied potential +50 millivolts. Signal analysis gave the current value at +18.5 picoamperes.

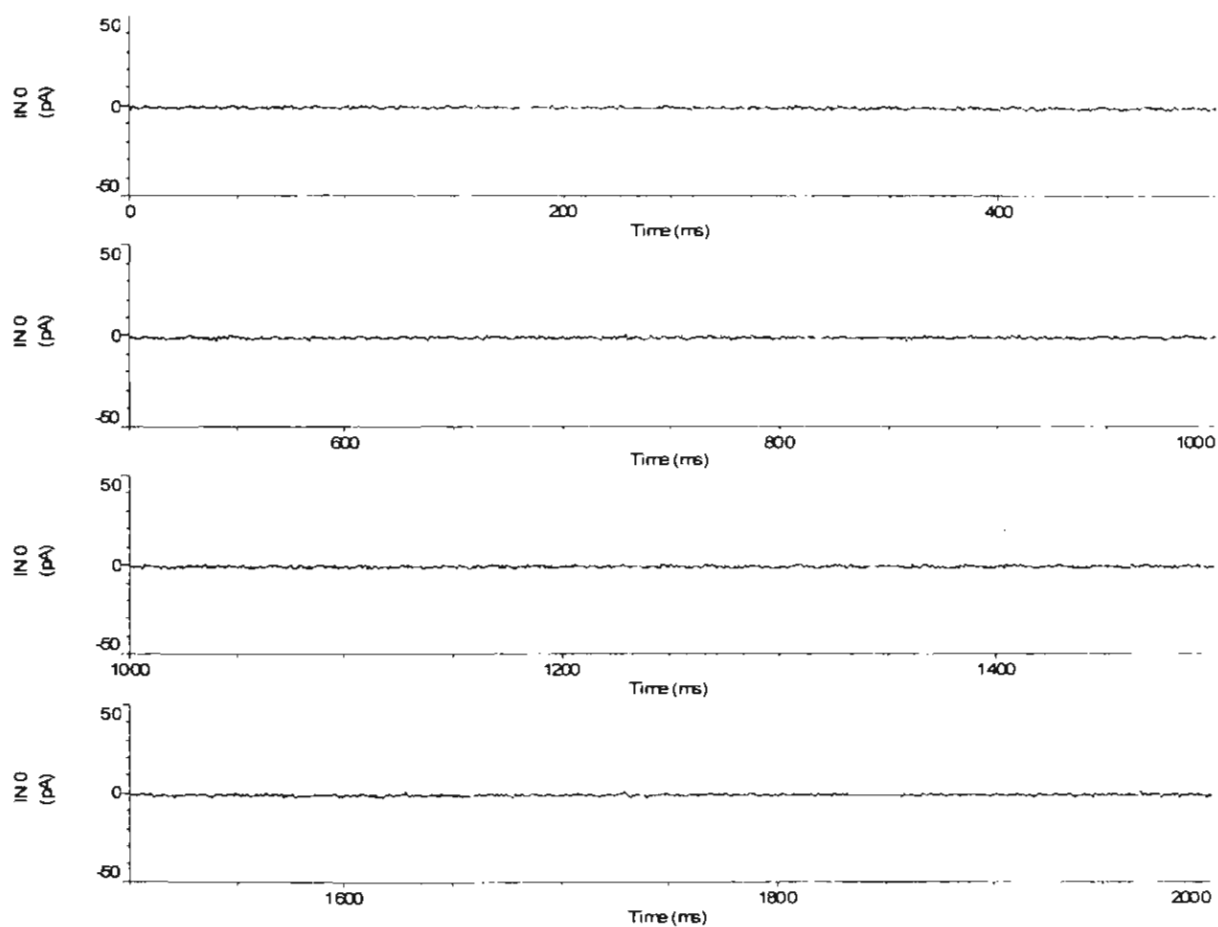


Figure 26: A plot between current and time for asymmetric planar lipid bilayer experiment at applied potential -20 millivolts. Signal analysis gave the current value at -0.9 picoamperes.

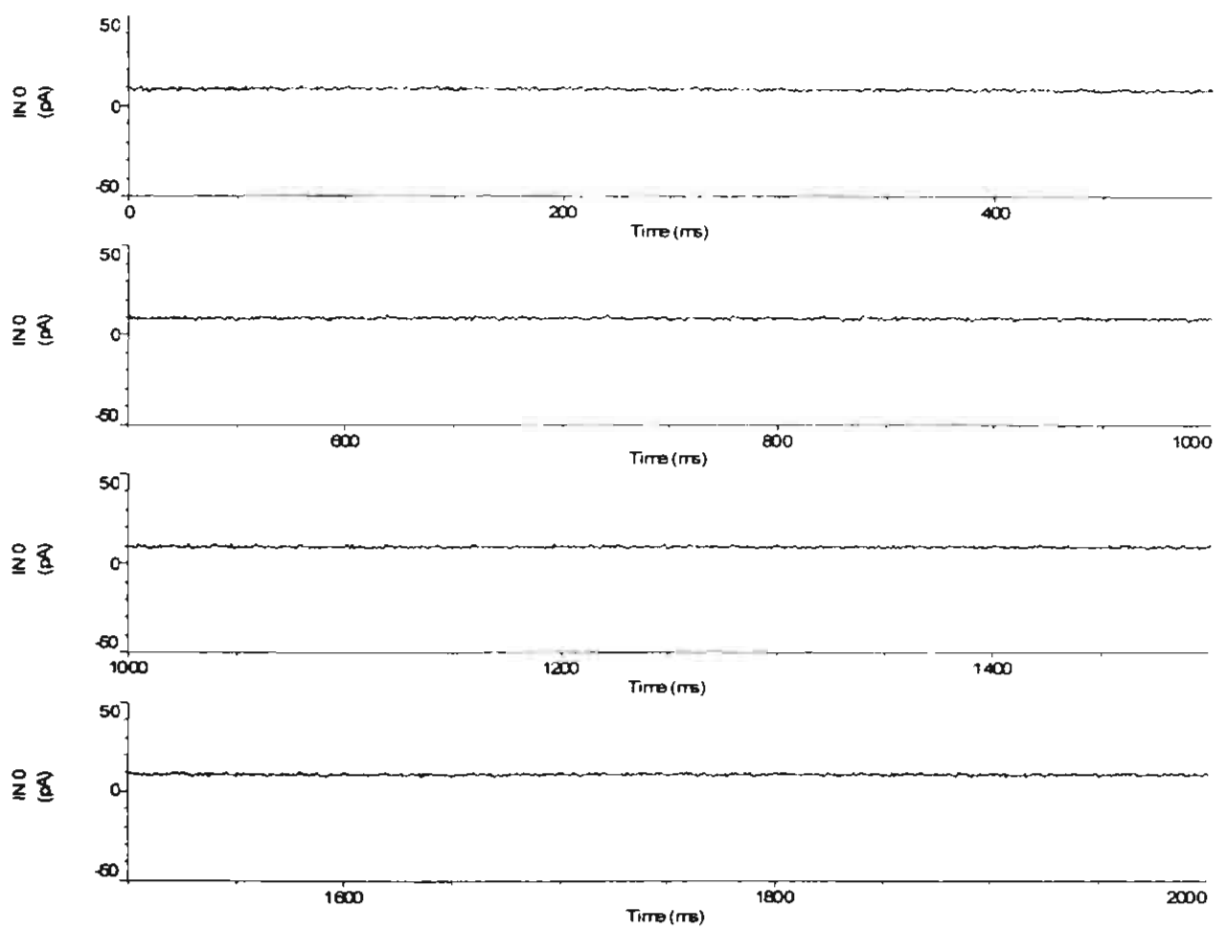


Figure 27: A plot between current and time for asymmetric planar lipid bilayer experiment at applied potential +20 millivolts. Signal analysis gave the current value at +9.6 picoamperes.

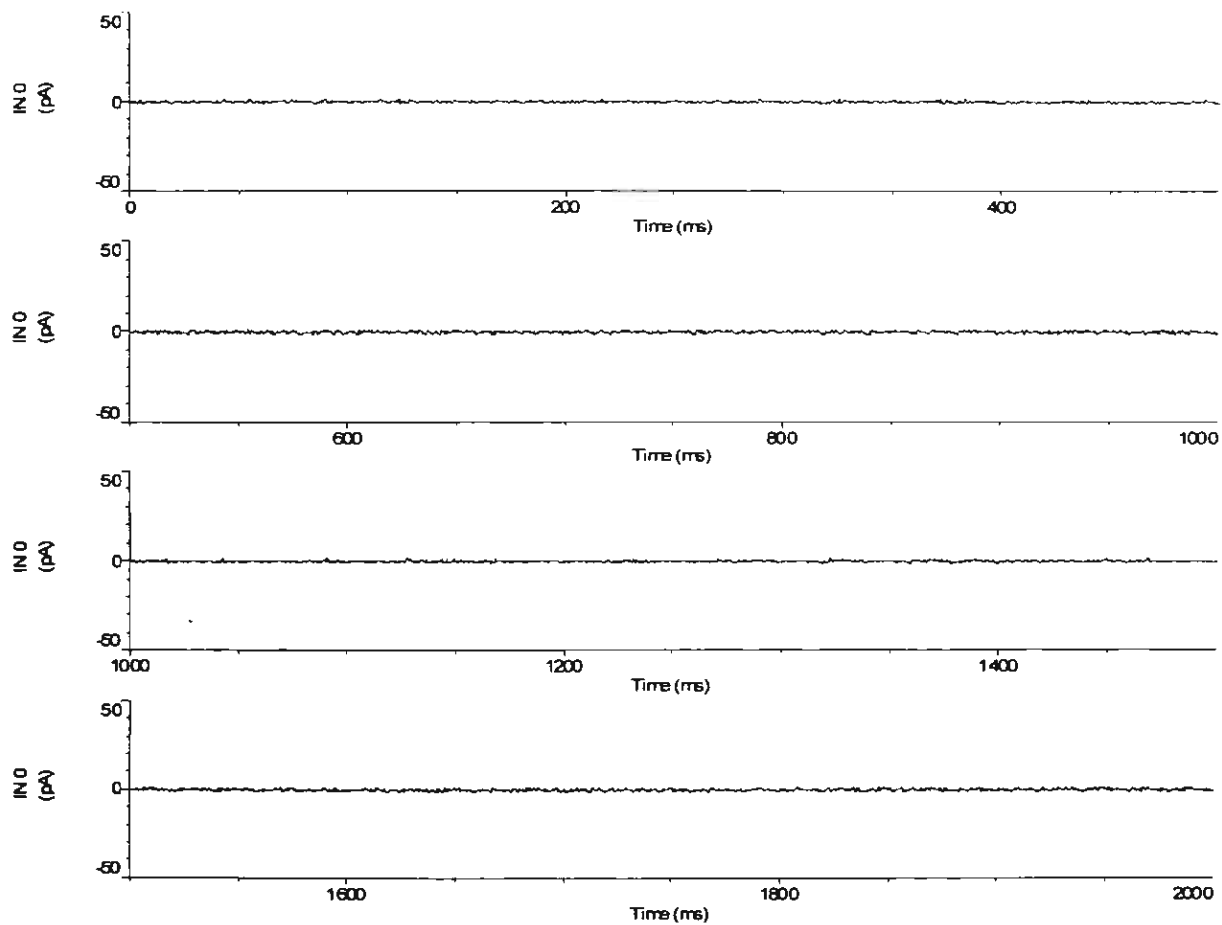


Figure 28: A plot between current and time for asymmetric planar lipid bilayer experiment at applied potential -18 millivolts. Signal analysis gave the current value at -0.4 picoamperes.

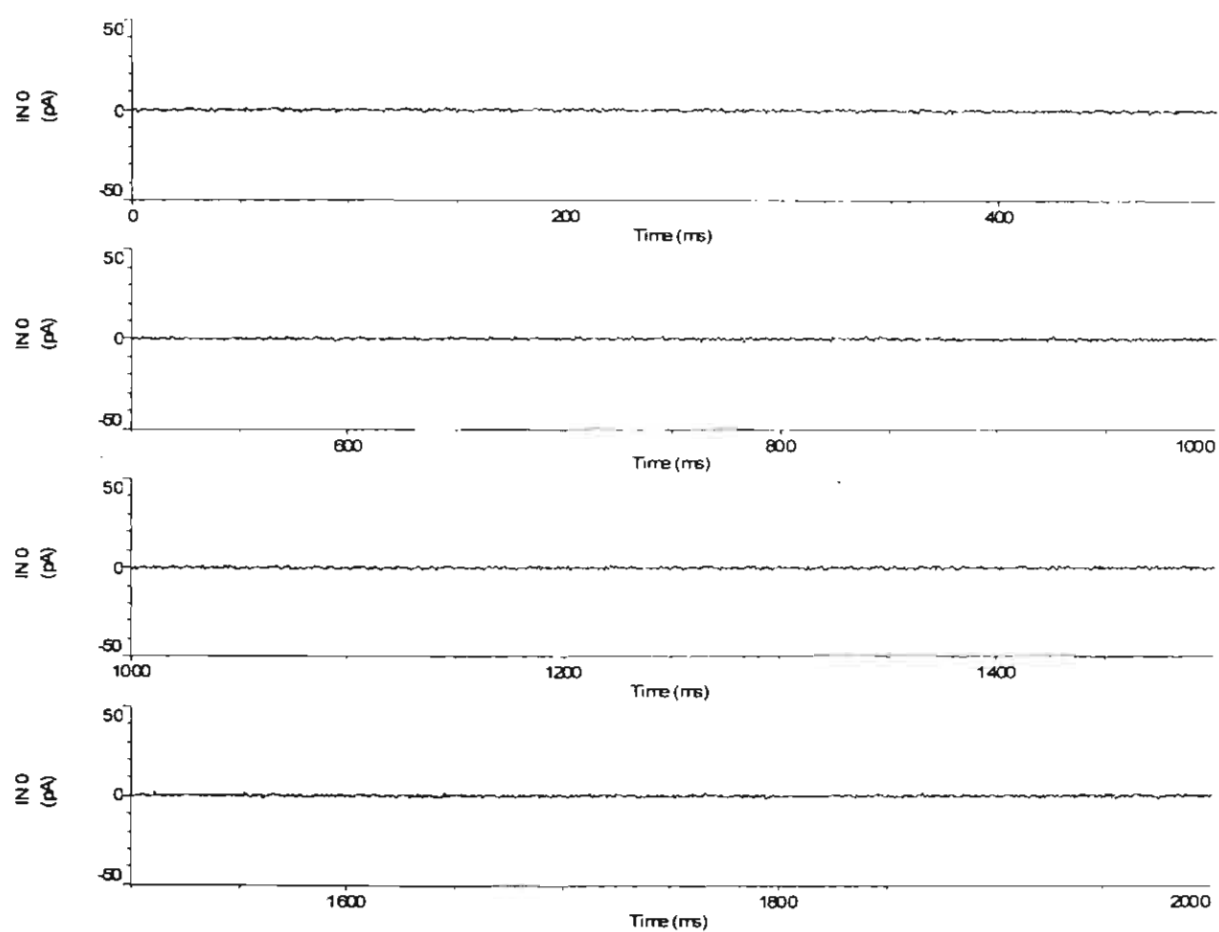


Figure 29: A plot between current and time for asymmetric planar lipid bilayer experiment at applied potential -16 millivolts. Signal analysis gave the current value at +0.2 picoamperes.

From the detected currents observed in various applied potential conditions, we can summarize the data as listed in Table 6.

Applied Potentials (mV)	Detected Currents (pA)
- 80	- 18.5
+ 80	+ 29.0
- 50	- 8.4
+ 50	+ 18.5
- 20	- 0.9
+ 20	+ 9.6
- 18	- 0.4
- 16	+ 0.2

Table 6: Detected current values at various applied potentials obtained from asymmetric planar lipid bilayer experiment.

A plot of detected currents against applied potentials has produced a linear relationship between the two variables (Figure 30). Curve fitting was performed with good correlation factor (R^2) of 0.997 and fitted equation of $I = 0.288V + 4.862$. Unlike a symmetrical mode, the asymmetrical experiment will not give a zero current at applied potential of 0 mV. To get a zero current in this system, there must be some potential applied for a reversal of an ion flow. This reversed potential can be determined from the fitted equation by substitution of I with zero. A reversed potential obtained for our PEP-4 peptide was -16.88 mV. Since a negative potential is needed to inhibit ion flow of K^+ ion, this channel is classified as a cation-selective channel.

From our planar lipid bilayer experiment the results revealed that the pore or channel can be generated on the artificial membrane by PEP-4 peptide. The characteristics of this channel are the conductance of 188 pS and selecting for cation. However other morphology and properties of this channel can not be characterized by planar lipid bilayer experiment.

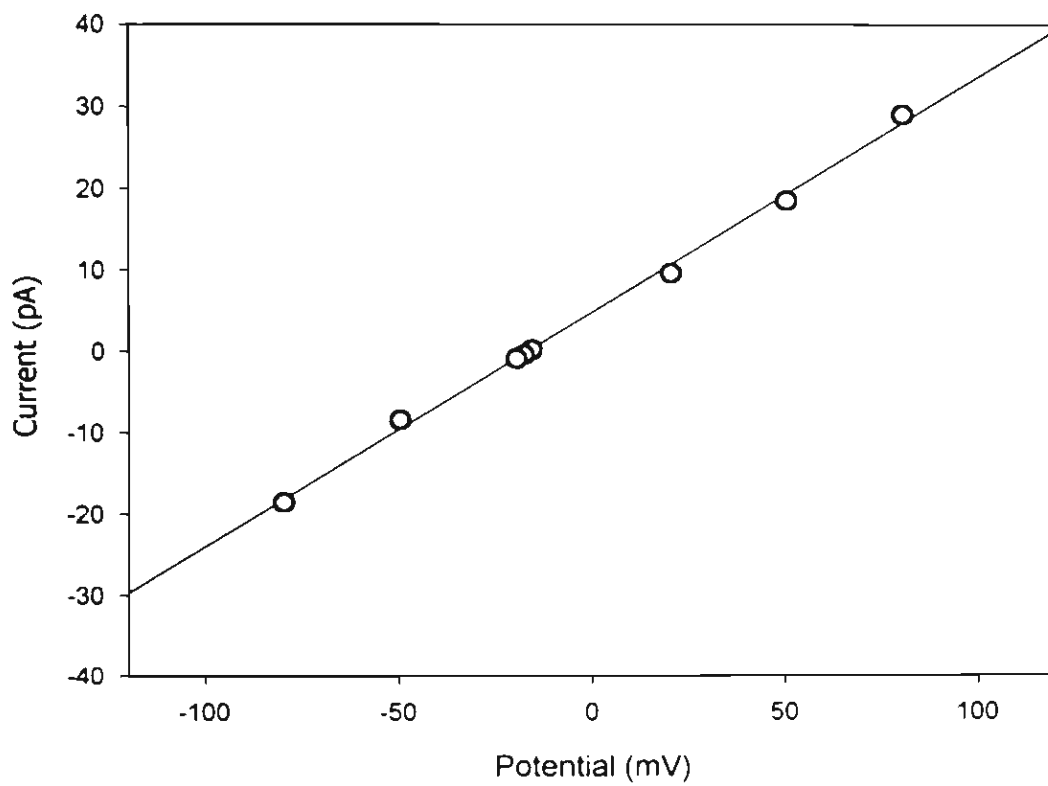


Figure 30: A plot between applied potentials and detected currents obtained from planar lipid bilayer experiment in asymmetrical mode.

Concluding Remarks

We have demonstrated a process for *de novo* design for an alpha-helical peptide with pore-forming activity. The design strategy was based on the selection of amino acid residue of high helical propensity, the minimal length of helix for membrane insertion, and the amphipathicity containing one-third of hydrophobic surface. The designed peptide models were analyzed and showed comparable stability with known helical peptide and then synthesized by solid phase synthesis. Characterization of the purified peptides indicated an alpha helical structure as expected. We found one peptide, PEP-4 of sequence Acetyl-YALSLAATLLKEAASL-OH, is capable of releasing glucose entrapped in the liposome. Further characterization of this peptide on an artificial membrane using planar lipid bilayer gave a channel activity for cation permeability with 188 pS conductance.

This work has proved to be an accomplishment of peptide design strategy based on a simple structural concept. However the application of the design principle may not be the universal rule for definite prediction of structure and function. It has rather been employed as a valuable facilitating tool for structural prediction and for more understanding on the concept of protein and peptide structure and function.

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Project Outputs

1. Publication using data from the project:

Krittanaï, C. and Panyim, S. A Channel-Forming Peptide from *De Novo* Design.
(*Manuscript is in preparation*)

2. Publication associated with data or techniques established by the project:

2.1 Puntheeranurak, T., Leetachewa, S., Katzenmeier, G., **Krittanaï, C.**, Panyim, S. and Angsuthanasombat, C. (2001) Expression of biochemical characterization of the *Bacillus thuringiensis* Cry4B alpha4-alpha5 pore-forming fragment. *J. Biochem. Mol. Biol.* 34: 293-298.

2.2 **Krittanaï, C.**, Lungchukiet, P., Ruagwetdee, S., Tuntitippawan, T., Panyim, S. and Angsuthanasombat, C. (2001) Redesign of an interhelical loop of the *Bacillus thuringiensis* Cry4B δ -endotoxin for proteolytic cleavage. *J. Biochem. Mol. Biol.* 34: 150-155

2.3 Jirajaroenrat, K., Pongjaroenkit, S., **Krittanaï, C.**, Prapanthadara, L. and Kettermann, A. J. (2001) Heterologous expression and characterization of alternatively spliced glutathione S-transferases from a single *Anopheles* gene. *Insect Biochem. Mol. Biol.* 31: 867-875.

3. Poster presentation in international conferences:

3.1 Uawithya, P., Krittanaï, C., Katzeimer, G., Leetachewa, S., Panyim, S., Angsuthanasombat, C. 3-D models for *Bacillus thuringiensis* Cry4A and Cry4B insecticide proteins based on homology modeling.
In the 32th Annual Meeting of Society of Invertebrate Pathology, University of California at Irvine, August 22-27, 1999

3.2 Uawithya, P., Chanama, U., Potvin, L., Schwartz, J.L., **Krittanaï, C.**, Katzenmeier, G., Panyim, S. and Angsuthanasombat, C. (2000) Ion-channel formation in artificial lipid membranes by the *Bacillus thuringiensis* Cry4B toxin.
In the 44th Annual Meeting for Biophysical Society, New Orleans, USA.
(Abstract Published in *Biophys. J.*, 78: 175.)

3.3 Puntheeranurak, T., Leetacheewa, S., Katzenmeier, G., **Krittanaï, C.**, Panyim S, Angsuthanasombat, C. Expression and biochemical characterisation of the *Bacillus thuringiensis* Cry4B A1-A5 pore-forming fragment.
In the 45th Annual Meeting for Biophysical Society, Boston, USA,
(Abstract published in *Biophys J.*, 80: 585.)