Spontaneous Oscillation of the Electrical Membrane Potential in Tri-Block Copolypeptide Membranes Composed of L-Glutamic Acid and L-Leucine

Norihiko Minoura,* Sei-ichi Aiba, and Yukihiko Fujiwara

Contribution from the Department of Organic Materials, National Institute of Materials and Chemical Research, Agency of Industrial Science and Technology, Higashi, Tsukuba, Ibaraki 305, Japan

Received July 20, 1992

Abstract: A membrane produced from a tri-block copolypeptide composed of poly(L-glutamic acid) as outer segments and poly(L-leucine) as an inner segment spontaneously generated electrical pulses under a concentration gradient of KCl in the absence of any external current, voltage, or pressure. At lower KCl concentrations the current-voltage curves of the membrane were sigmoidal, and hysteresis appeared. This result suggests that the membrane can have two different resistance states which are related to α-helix and random-coil conformations of the poly(L-glutamic acid) chain. According to the circular dichroism and FT-IR spectra of the membrane, in the higher resistance state the poly(L-glutamic acid) chains exhibit random-coil structure, while in the lower resistance state the chains exhibit α-helix structure. The oscillation mechanism of the membrane potential may be explained by a periodic change of KCl permeability of the membrane, which is induced by a periodic change in the conformation between α-helix and random-coil structure of the poly(L-glutamic acid) chain. The frequency of the electrical pulses was pH dependent and showed a maximum between pH 3 and pH 4.

Introduction

Electrical oscillatory phenomena have been investigated in various kinds of artificial membranes.[1] In particular, membranes composed of polypeptides are of special interest because they serve as a model for membrane proteins. Under a dc electric field, Shashoua[2] observed oscillations of a polyelectrolyte membrane formed by interfacial precipitation of poly(glutamic acid) and polylysine. Following such studies, Spangler et al.[3] undertook similar experimental and theoretical studies of poly(glutamic acid)–Ca^{2+} membranes and proposed a model for the oscillatory phenomena. The model was based on a change of polymeric conformation induced by salt accumulation at the interfacial region between polyelectrolyte layer and Ca^{2+}-chelated neutral layer inside the membrane during a current flow. The oscillation was accounted for by a periodic change of the membrane permeability accompanied by this structural change.

The present paper reports the observation of spontaneous oscillation of the electrical membrane potential, generated across a tri-block copolypeptide membrane separating KCl solutions of different concentrations without any additional external forces (e.g., pressure, voltage, electrical current).

Experimental Section

Synthesis of Block Copolypeptide. *N*-Carboxyanhydride (NCA) of γ-benzyl-L-glutamate and L-leucine was synthesized by reacting γ-benzyl-L-glutamate or L-leucine, respectively, with phosgene in a dioxane solution.[4] The benzyl group in γ-benzyl-L-glutamate protects the carboxyl group of L-glutamic acid during the polymerization reaction.

Preformed polypeptide blocks containing free amino groups can act as initiators for polymerization of the peptide chain with other α-amino acid NCAs, so that copolypeptides containing blocks of amino acids can be obtained.[5] However, monoamine initiators produce ABA-type block copolymers with end-blocks of unequal length. To make a symmetrical ABA-type block copolypeptide, the center block was initially prepared using a diamine initiator, followed by simultaneous polymerization of the end blocks.

The polymerization conditions for (γ-benzyl-L-glutamate)$_x$-(L-leucine)$_y$-(L-leucine)$_y$-(γ-benzyl-L-glutamate)$_x$ tri-block copolypeptide are as follows: The L-leucine block was synthesized using the L-leucine-NCA in benzene–dioxane (19:1, by volume) solution with 1,6-hexamethylenediamine as the initiator. The ratio of the L-leucine-NCA to the initiator, [B]/[I], was 1000. The polymerization reaction was monitored by measuring the carbon dioxide that evolved. After 3 days the reaction reached more than 90% conversion. On completion of the polymerization of the L-leucine blocks, γ-benzyl-L-glutamate-NCA dissolved in benzene–dioxane solution was added to the polymerization solution. In this solution, the ratio of the γ-benzyl-L-glutamate-NCA to the initiator, [A]/[I], was 600. A total polymerization time of 14 days was allowed. All polymerizations were carried out at room temperature at a concentration of 20 g/L relative to the benzene–dioxane solution. The copolymer was precipitated by pouring the polymerization solution into methanol and then washed several times with methanol. To remove coexisting homopolymers, poly(L-leucine) and poly(γ-benzyl-L-glutamate), the mixture was fractionated using chloroform and trifluoroacetic acid. The product was then dried under vacuum for 5 days. Monomer composition of the block copolypeptide was estimated by elemental analysis and high-resolution NMR spectroscopy (measured in trifluoroacetic acid). Mole fractions were $x = 0.18$ and $y = 0.32$. Thus, the measured monomer composition was almost the same as the loading ratio of L-leucine-NCA and γ-benzyl-L-glutamate-NCA.

Preparation of Membrane. Thin membranes (about 0.018 mm thick) were cast on a glass plate from a benzene solution (0.5%) of the block copolypeptide and dried by slow evaporation of the solvent at 20 °C with subsequent drying under vacuum at 80 °C for 5 days. After this procedure the block copolypeptide membrane was hydrolyzed in a solvent mixture consisting of methanol–2-propanol–5 M NaOH aqueous solution (2:2:1, by volume) for 20 days at 18 °C to eliminate the carboxyl-protecting groups. The hydrolyzed membrane was thoroughly washed with methanol

(1) (a) Teorell, T. *Z. Physik. Chem. Neue Folge* **1958**, *15*, 385. (b) Seno, M.; Yamabe, T. *Bull. Chem. Soc. Jpn.* **1963**, *36*, 877. (c) Monnier, A. M.; Monnier, A. *J. Physiol. (Paris)* **1964**, *56*, 410. (d) Mueller, P.; Rudin, D. O. *Nature* **1967**, *213*, 603. (e) Kobatake, Y. *Adv. Chem. Phys.* **1975**, *29*, 319. (f) Yoshikawa, K.; Matsubara, Y. *J. Am. Chem. Soc.* **1984**, *106*, 4423. (g) Ishii, T.; Kuroda, Y.; Omochi, T.; Yoshikawa, K. *Langmuir* **1986**, *2*, 319.
(2) Shashoua, V. E. *Faraday Symp. Chem. Soc.* **1974**, *9*, 174.
(3) Huang, L. M.; Spangler, R. A. *J. Membrane Biol.* **1977**, *36*, 311.
(4) Fuchs, F. *Ber.* **1922**, *55*, 2943.
(5) Hayashi, T.; Walton, A. G.; Anderson, J. M. *Macromolecules* **1977**, *10*, 346.

0002-7863/93/1515-5902$04.00/0 © 1993 American Chemical Society

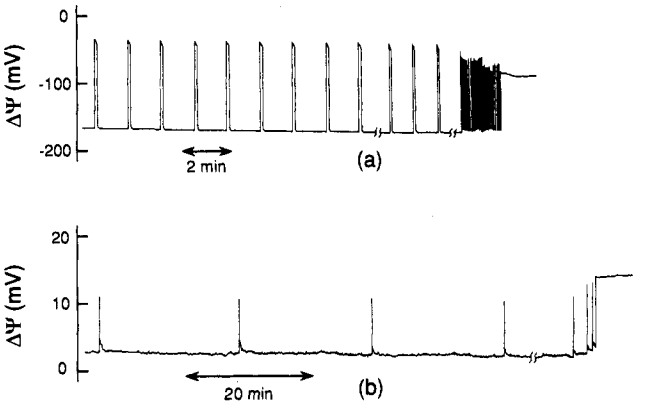

Figure 1. Oscillations of electrical membrane potential across a block copolypeptide membrane between solutions of 1.0 mM KCl and 1.0 M KCl: (a) at pH 5.4 and (b) at pH 2.0.

and then dried in air. Thus, a tri-block copolypeptide membrane of L-glutamic acid and L-leucine for the membrane potential studies was obtained.

Fourier Transform Infrared Absorption (FT-IR) Spectra. To study molecular conformation of the tri-block copolypeptide membrane, attenuated total reflectance infrared (ATR) was employed. The ATR apparatus was a WILKS model with a germanium internal reflection element set at an incident angle of 45°. The spectrometer (Nicolet 20DXB Fourier Transform Infrared) was equipped with an MCT detector. The gain setting was 16 at 2 cm^{-1} resolution. One thousand scans were collected for each spectrum.

Prior to measurement, membranes were immersed in KCl solution for a few days. After removing from the solution, the membrane was wiped dry with filter paper and mounted in the ATR apparatus.

Circular Dichroism (CD) Spectra. For the CD spectra a very thin membrane (about 0.001 mm thick) is necessary. This was achieved by casting a benzene solution of the block copolypeptide on a quartz plate (60 mm × 10 mm, 1 mm thick). Drying and hydrolyzing was done by the same procedure as that for the membrane used in membrane potential studies. The CD spectra of the membrane were measured by placing the quartz plate attaching the membrane in a quartz cell (optical path length, 10 mm) filled with KCl solution, using a JASCO J-600 spectropolarimeter.

Membrane Potential. The membrane potential experiments were carried out at 25 °C in an acrylic resin cell composed of two compartments of equal volume (270 mL). A membrane was clamped in place between these compartments. Then 1.0 M KCl was added to one compartment and 1.0 mM KCl to the other. The following electrolyte systems were used to vary pH: KCl–HCl and KCl–KOH. The pH values of the solutions in both compartments were equal and remained constant during the experiment. The potential difference across the membrane was measured with an electrometer (Hokuto Denko Co., Ltd., Japan, Type HE-101A; inner resistance $10^{11}\Omega$) connected to two calomel electrodes and monitored with a recorder (Rikadenki Co., Ltd., Type R-53).

Current Voltage Measurement. Using the acrylic resin cell from the membrane potential measurements, the current-voltage curves were measured as a function of salt concentration at 25 °C. In these experiments the concentrations of the solutions in both compartments were equal. The potential difference across the membrane was measured with an potentiostat/galvanostat (Nikko Keisoku Co., Ltd., Japan, Type NFG-3) including a function generator. It was connected to two pairs of Ag/AgCl electrodes: one of the pairs was used for the detection of the potential difference across the membrane; the other pair was for the current supply.

Electron Microscopy. The domain structure of the block copolypeptide membrane (stained by osmium tetroxide) was observed by a Nippon Denshi Transmission Electron Microscope JEM-1000 CE. Ultrathin sections of about 35-nm thickness were cut perpendicular to the membrane surface by an ultramicrotome.

Results and Discussion

Membrane Potential. Figure 1 shows electrical membrane potentials for the block copolypeptide membrane placed between the solutions of 1.0 mM KCl and 1.0 M KCl. In Figure 1a, the changes of the electrical potential started after a certain induction period (about 20 h) and continued for about 20 h before dying

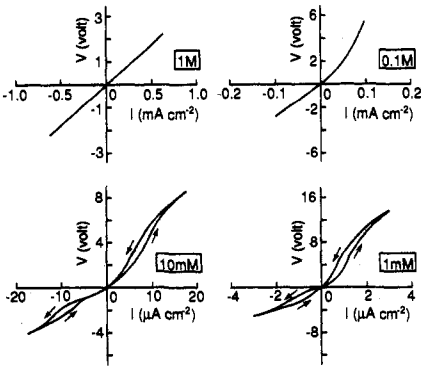

Figure 2. Current-voltage curves at various KCl concentrations (pH 5.8).

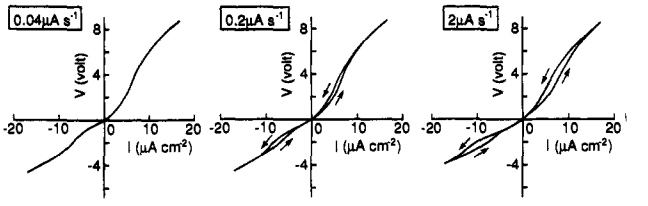

Figure 3. Current-voltage curves at various current scan speeds (10 mM KCl, pH 5.8).

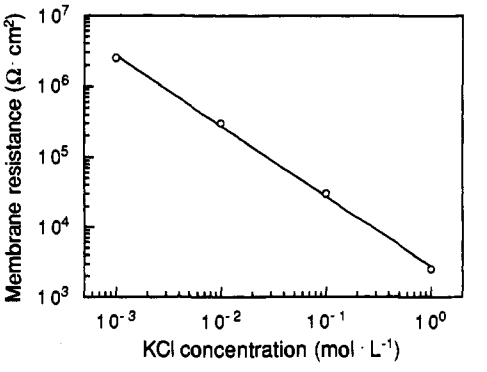

Figure 4. Membrane resistance as a function of KCl concentration.

out. The amplitude of the oscillations was 135 mV and remained nearly constant during the oscillatory period. The period between the pulses was 80 s, and the width of a pulse was 6 s. In the final stage of the oscillatory period (about 20% of the whole oscillatory period time), the distance between the pulses became shorter, and bursting-like oscillations were observed.

Figure 1b shows an example of a low-frequency oscillation. The period between the pulses was 21 min. In this case the membrane was placed between the solutions of 1.0 mM KCl and 1.0 M KCl both of which were exactly adjusted to pH 2.0 with concentrated hydrochloric acid. The whole figure shown in Figure 1b is similar to that shown in Figure 1a.

Membrane Resistance. Figure 2 shows the current-voltage (I-V) curves at various KCl concentrations (pH 5.8). In a 1.0 M KCl solution, ohmic behavior was observed. However, the I-V curve was concave upward in the case of 100 mM KCl solution. In the lower KCl concentration, the I-V curves were sigmoidal, and hysteresis appeared. When the scan speed of the current was lowered, the hysteresis disappeared, but the sigmoidal shape still remained (Figure 3). Relaxation of the molecular conformations may be responsible.

The membrane resistance was calculated from the slope near zero of the I-V curve and is shown in Figure 4 as a function of KCl concentration. The membrane resistance increased remarkably with decreasing KCl concentration.

Morphology of the Membrane. Figure 5 shows an electron micrograph of an ultrathin section cut perpendicular to the surface of the block copolypeptide membrane. The dark portions

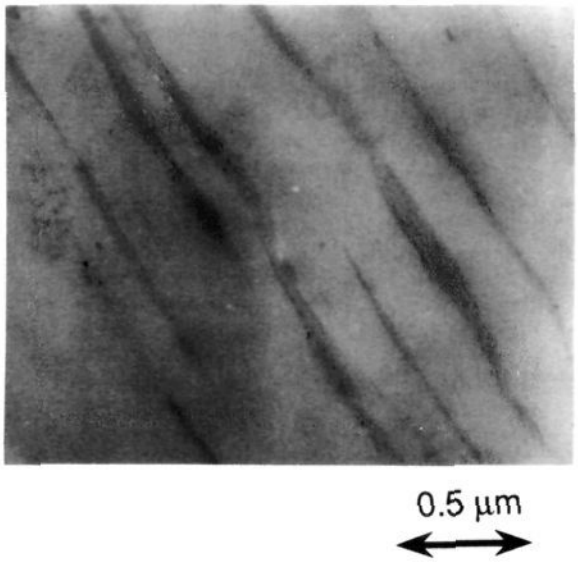

Figure 5. Electron micrograph of ultrathin section of leucine–glutamic acid block copolypeptide membrane (cut vertically to the surface).

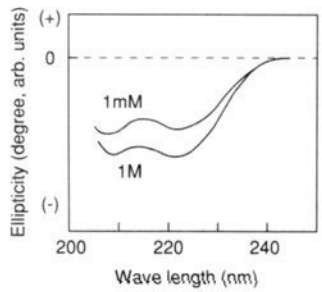

Figure 6. Circular dichroism spectra of membrane in KCl solutions of different concentration.

correspond to the domains composed of the poly(L-glutamic acid) block chains embedded in a continuous matrix of the poly(L-leucine) phase. The domains are hydrophilic and the matrix is hydrophobic. The structure of the hydrophilic domains is nearly cylindrical, extending from one surface to the other, which permit water-soluble solutes (KCl, NaCl, LiCl, glucose) to permeate through the membrane.[6] The domain size (diameter 100 nm) and the distance between two adjacent domains (140 nm) were estimated from many transmission electron micrographs.[7]

Assuming the hydrophilic domains to be perfect cylinders, the volume fraction of the domain was calculated to be 46%. This value is in good agreement with the theoretical expectation of 48%,[7] calculated from the chemical composition of the copolypeptides and the densities of the membranes composed of the corresponding homopolymers, assuming additivity rule for their volumes can be applied. These results suggest that the morphological structure described above exists in the membranes.

Molecular Conformation of the Membrane. The conformational change of the poly(L-glutamic acid) chains of the block copolypeptide in the membrane was observed by circular dichroism studies. Figure 6 shows the CD spectra of the membrane immersed in KCl solutions of different concentrations (pH 5.6). A membrane immersed in a 1.0 M KCl solution had higher helix content than in a 1.0 mM KCl solution, because the value of ellipticity at 222 nm is lower.[8] Such spectral changes caused by the alteration of KCl concentration were reversible. This

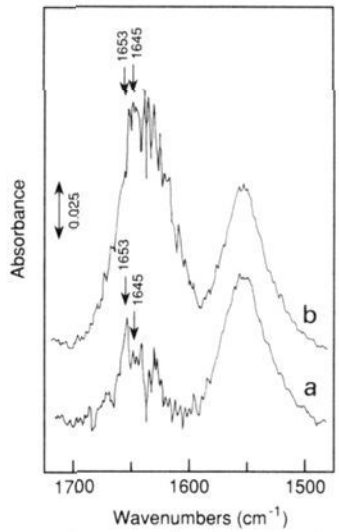

Figure 7. ATR-FT-IR spectra of poly(L-glutamic acid) domains. Membranes were immersed in 1.0 mM KCl solution (a) and 1.0 M KCl solution (b). These spectra were obtained by subtracting a spectrum of poly(L-leucine) homopolymer from each spectrum of the tri-block copolypeptide.

reversible conformational change from α-helix to random coil is presumed to occur in the domains consisting of the poly(L-glutamic acid) block.

The infrared absorption measurements gave similar results. Figure 7 shows the FT-IR spectra of the poly(L-glutamic acid) chains of the membranes immersed in KCl solutions of different concentrations (pH 5.5). These spectra were obtained by subtracting the poly(L-leucine) homopolymer spectrum from that of the tri-block copolypeptide. After immersing the membrane in 1.0 mM KCl solution, absorption indicative of random-coil conformation[9,10] was found at 1653 cm^{-1} (Figure 7a). After immersing the same membrane in 1.0 M solution, a new absorption, characteristic of α-helix conformation,[9,10] appeared at 1645 cm^{-1} in additiion to the absorption bands of the random-coil conformation (Figure 7b).

Since the increase of KCl concentration causes the increase of the helix content as shown in Figures 6 and 7, a larger helix content in the membrane may be responsible for a lower membrane resistance in Figure 4.

Mechanism of Oscillations. According to the model for the oscillatory phenomena proposed by Spangler et al.,[3] the oscillation was accounted for by an iteration of accumulation and release of salt concentration inside membrane coupled with a change of polymeric conformation.

In order to explain the oscillation mechanism in our block copolypeptide membrane, we have to take into account the conformational change of the poly(L-glutamic acid) chains. The sigmoidal curves shown in Figures 2 and 3 suggest that two resistance states exist in the membrane when the membrane is exposed to the lower KCl concentration. This is shown schematically in Figure 8. In the lower resistance state (R1) the membrane has a larger helix content than that in the higher resistance state (R2). That is, ions can permeate more easily through α-helix chains than they can through random-coil chains.

A plausible mechanism for the oscillation of the membrane potential is shown schematically in Figure 9. It may be explained qualitatively as follows: Prior to the oscillation experiment, the membrane was washed with pure water. At this initial stage,

(6) Minoura, N.; Aiba, S.; Fujiwara, Y. *J. Appl. Polym. Sci.* **1986**, *31*, 1935.
(7) Manuscript in preparation.
(8) Fasman, G. D.; Hoving, H.; Timasheff, S. N. *Biochemistry* **1970**, *9*, 3316.
(9) Miyazawa, T. In *Poly-α-Amino Acids: Protein Models for Conformational Studies*; Fasman, G. D., Ed.; Marcel Dekker: New York, 1967; pp 69–103.
(10) Bamford, C. H.; Elliott, A.; Hanby, W. E. In *Synthetic Polypeptides: Preparation, Structure, and Properties*; Academic Press: New York, 1956; pp 143–167.

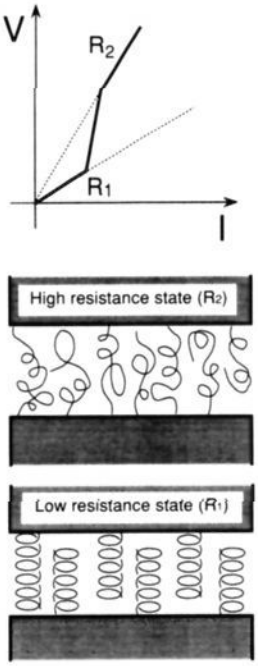

Figure 8. Relation between membrane resistance and the secondary structure of poly(L-glutamic acid) block chains in the domains.

poly(L-glutamic acid) chains have random-coil structure because of the electrostatic repulsion between the carboxyl groups of glutamic acid. After one side of the membrane was exposed to 1 M KCl and the other side to 1 mM KCl, KCl starts to penetrate into the membrane and the conformation of the poly(L-glutamic acid) chains changes to α-helix at the higher concentration side (induction period). This is because the α-helix content increases with increasing KCl concentration (Figures 6 and 7). Thus, the helix content in the membrane gradually increases with time, as the KCl concentration in the membrane increases from the high concentration side to the low concentration side by salt penetrating into the membrane (channel close state, in Figure 9). If the concentration inside the membrane is high enough that even the poly(L-glutamic acid) chains exposed to the low concentration side change the conformation to α-helix, then we have the open state (channel open state, in Figure 9). This open state is only very short-lived because the diffusivity of the α-helical form is higher and so the ions penetrate out of the membrane into the low concentration compartment. This lowers the KCl concentration inside the membrane. As a result of the reduced concentration, the conformation changes back to random coil (channel close state, in Figure 9).

Again accumulation of KCl begins in the membrane, and then the conformational change to helix occurs in the surface exposed to 1.0 mM KCl (channel open state). Thus open and closed channel states alternate repeatedly. The periodic change of the conformation can give rise to a periodic change of KCl permeability of the membrane. As a result, the membrane potential is observed to change periodically.

The interdependence of these processes can be illustrated in a simple manner:

change of salt concentration inside membrane $\Leftarrow$
$\Downarrow$ $\Uparrow$
conformational change of polymeric molecule $\Uparrow$
$\Downarrow$ $\Uparrow$
change in membrane permeability $\Rightarrow$ $\Rightarrow$

If the processes are coupled in such a way that sufficient feedback is built in, they will continuously reinforce themselves and the system can become unstable.

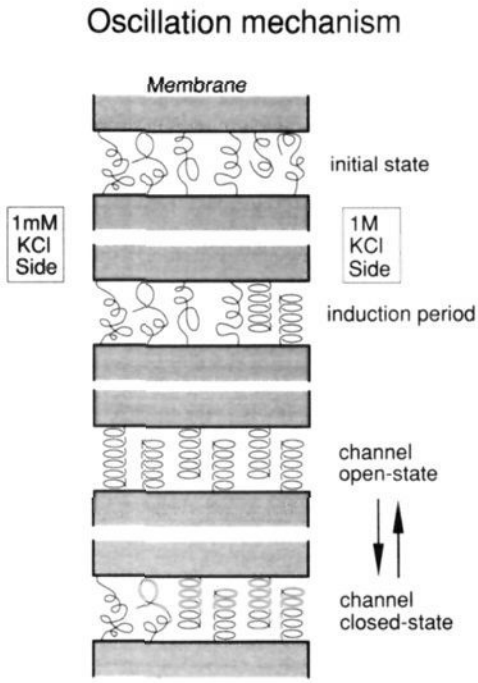

Figure 9. A proposed mechanism for the oscillation of the membrane potential.

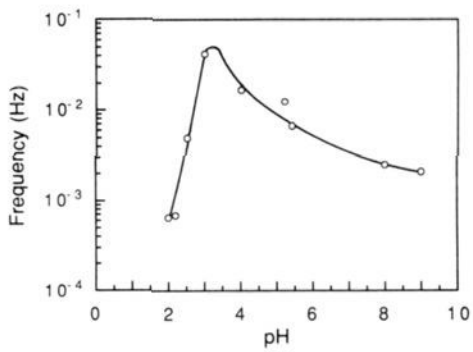

Figure 10. Dependence of the frequency of the electrical oscillation on the pH of the solution.

pH Dependence of the Frequency of Oscillations. If the spontaneous oscillation is accounted for by an iteration of accumulation and release of salt concentration in the domains of the membrane coupled with the conformational transition, the period between pulses of the oscillation should be controlled by changing the pH of the external KCl solution because the helix-coil transition of the poly(L-glutamic acid) chains is affected by the pH of the solution.[6] In fact at pH 2.0 a low-frequency oscillation was observed, as shown in Figure 1b. This experimental result suggests that the period between the pulses depended on the pH of the solution. A dependence of the frequency of the electrical oscillations on the pH of the solution is plotted in Figure 10. The frequency showed a maximum between pH 3 and pH 4 and sharply decreased in the lower pHs.

The high frequency between pH 3 and pH 5 shown in Figure 10 may be due to the ease of helix-coil transition of the poly(L-glutamic acid) chain induced by salt concentration in this pH range. Since in the lower pH (<pH 2) α-helix form is stable and in the higher pH (>pH 9) random coil form is stable, in these pH ranges the helix-coil transition may not be triggered by the salt concentration. Thus, the periodic change of the membrane potential was found only in the pH range of pH 2–9.

In a cross-linked poly(L-glutamic acid) membrane, which can change conformation, we could not find electrical pulses. It seems that the domain structure surrounded by the hydrophobic poly(L-leucine) phase is important for the generation of electrical pulses. If the hydrophilic domains were swollen by the conformational change, KCl would be allowed to pass continuously. The hydrophobic matrix may prevent the membrane from swelling. Thus only conformational changes enable KCl to periodically pass through the membrane.

Conclusion

A tri-block copolypeptide membrane composed of L-glutamic acid and L-leucine exhibited oscillatory phenomena under the concentration gradient of KCl without any additional external forces. A mechanism for the oscillation is explained qualitatively in relation to a conformational transition between α-helix and random-coil structures of the poly(L-glutamic acid) block chains.