Mechanisms of Membrane Fusion

By

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Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy for the University of London, 1993.
This thesis is concerned with studies on two aspects of membrane fusion. The first aspect is the mechanism of membrane fusion in electrically-induced cell fusion. The second aspect is the mechanism of membrane fusion induced by fusogenic viral peptides.

Evidence was found from both fluorescence microscopy and freeze-fracture electron microscopy for the occurrence of hemi-fusion in the electrofusion of human erythrocytes. The conditions that favour hemi-fusion as opposed to complete fusion were characterised, and the possibility that hemi-fusion might precede complete electrically-induced cell fusion are discussed.

The procoagulant activity of human erythrocytes, which provides a measure of the translocation of phosphatidylserine from the inner to the outer monolayer of the plasma membrane, has been compared with the percentage cell fusion in experiments on erythrocyte fusion induced by electrical breakdown pulses under differing experimental conditions. It seems possible that a localised, surface exposure of phosphatidylserine may contribute to the "long-lived fusogenic state".

Divalent cations in the pulsing medium may interact with phosphatidylserine molecules, translocated from the inner to the outer monolayer of the erythrocyte plasma membrane by breakdown pulses, to stabilise the pulsed erythrocyte membrane against haemolysis, and to assist the formation of pearl chains of pulsed cells. It was found that the entry of sugar molecules, via electropores in the plasma membrane, facilitated the
rounding-up of electrically fused erythrocytes into giant cells, while impermeable molecules e.g. poly(ethylene glycol) or dextran inhibited this process.

The secondary structures and orientations of the fusion peptides (corresponding to the N-terminus of HA2 protein of strains A/PR/8/34 and X31 influenza viruses (HA and WT peptide, respectively), a peptide (G1E peptide) with a substitution of glutamic acid for the glycine residue at the N-terminal of the WT peptide, and the fusion peptide corresponding to the N-terminus of gp41 protein of ARV2 strain HIV virus were investigated in egg PC phospholipid bilayers with polarized, attenuated total reflection Fourier transform infrared spectroscopy (ATR FT-IR). Both dried and hydrated samples were studied. The relationships between the observed secondary structures and orientation of these fusion peptides and how they may induce membrane fusion are discussed.
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Dr. Murata of Kyoto University kindly provided the HA peptide. The WT and G1E peptides were a gift of Dr. Wharton of National Institute of Medical Research, Mill Hill. The HIV1 peptide was kindly provided by Dr. Bansal of King's College, London. The preliminary observations on hemi-fusion, and the freeze fracture work presented here, were made by Dr. Ahkong, and I am grateful for the advice he has offered during the course of this work. Dr. D.Georgescauld of CNRS, France was responsible for measuring the diffusion coefficient of the DiI probe in electrofused erythrocytes.

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ABBREVIATIONS USED

AC - alternating current
ANTS - aminonaphthalene-trisulfonate
ATR - attenuated total reflection
BHA - bromelain released influenza hemagglutinin
CD - circular dichroism
CHO - Chinese hamster ovary
DC - direct current
DCl - deuterium chloride
D/H - deuterium/hydrogen
DMSO - dimethyl sulfoxide
DPA - dipicolinic acid
DPX - xylene-bis-pyridinium bromide
DPPC - dipalmitoyl phosphatidylcholine
FTIR - Fourier transform infrared
Ge - germanium
HA - hemagglutinin
HCl - hydrochloric acid
Hepes - N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IR - infrared
NBD-DOPE - N-(7-nitro-2,1,3-benzoxadiazol-4-yl)-dioleoylphosphatidylethanolamine
PBS - phosphate buffered saline
PC - phosphatidylcholine
PE - phosphatidylethanolamine
PI - phosphatidylinositol
PS - phosphatidylserine
Rhodamine-DOPE - N-(lissamine rhodamine B sulfonyl)-dioleoylphosphatidylethanolamine
s/n ratio - signal-to-noise ratio
Tm - temperature of the main phase transition in phospholipids
TFA - trifluoroacetic acid
WT - wild type
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Chapter One

Introduction

The aim of this thesis is to study the mechanism of membrane fusion in electrically-induced cell fusion and the mechanism of membrane fusion induced by fusogenic viral peptides.

As cell and membrane fusion processes are closely related to membrane structure and functions, this introduction therefore covers the structure and function of biomembranes. A review of membrane fusion is also provided. The introductions to electrofusion and influenza and HIV virus-induced fusion processes are included in Chapter Two and Chapter Five respectively. Since Fourier transform infrared (FT-IR) techniques are used to study the interaction of fusogenic viral peptides with lipid membranes in this thesis, the basic mechanism and application of FT-IR techniques in studying membrane structure and function are introduced in Appendix I.

1.1 Biomembranes

A plasma membrane encloses every cell, defining the cell's extent and maintaining the essential differences between its contents and the environment. The membrane is a highly selective filter and a device for active transport. It generates differences in ion concentrations between the interior and exterior of the cell.

All biological membranes are assemblies of lipid and protein molecules held together mainly by noncovalent interactions. Cell membranes are dynamic, fluid
structures, and most of their lipid and protein molecules are able to move about in the plane of the membrane. The lipid bilayer provides the basic structure of the membrane and serves as a relatively impermeable barrier to the passage of most water-soluble molecules. The protein molecules, are usually "dissolved" in the lipid bilayer, and mediate most functions of the membrane (Singer & Nicholson, 1972).

1.1.1 Bilayer formation

Lipid molecules are insoluble in water but dissolve readily in organic solvents. They constitute about 50% of the mass of most animal cell plasma membranes, nearly all of the remainder being from proteins. There are approximately $5 \times 10^6$ lipid molecules in a $1 \mu m \times 1 \mu m$ area of lipid bilayer. The three major types of lipids in cell membrane are phospholipids (the most abundant), cholesterol, and glycolipids. All three are amphiphilic. A typical phospholipid molecule has a polar group and two hydrophobic acyl chains. The acyl chains can differ in length, and in the degree and position of double bonds. Saturated acyl chains are composed of trans isomers. A double bond creates a kink in the tail. Differences in tail length and saturation are important because they influence the ability of phospholipid molecules to pack against one another and thereby affect the fluidity of the membrane. Most phospholipids and glycolipids form bilayers spontaneously in aqueous environments. These lipid bilayers tend to close on themselves to form sealed compartments. Compartments formed by lipid bilayers tend to reseal themselves when they are torn (Alberts et al., 1989).

1.1.2 Membrane fluidity

Individual lipid molecules are able to diffuse freely in the lipid bilayers. Lipid molecules in bilayers rarely migrate "flip-flop" from the monolayer on one side to that on the other (Rothman & Lenard, 1977). However, lipid molecules readily exchange
places with their neighbours within a monolayer (~10^7 times a second) (Devaux, 1991).

Individual lipid molecules rotate very rapidly about their long axes. Their hydrocarbon chains are flexible. The greatest degree of flexion occurring near the centre of the bilayer and the smallest adjacent to the polar head group.

Fluidity is one of the most important characteristics that make lipid bilayer an ideal structure for cell membranes. The precise fluidity of the plasma membrane is biologically important.

A synthetic lipid bilayer made from a single type of phospholipid changes from a liquid state to a rigid crystalline (gel) state at characteristic temperature. This change of state is called a phase transition, and the temperature (main phase transition temperature, T_m) at which it occurs is lower if the hydrocarbon chains are short or have double bonds. In synthetic bilayers containing a mixture of phospholipids with varying degrees of saturation (hence with different individual phase transition temperature), phase separation can occur. Individual phospholipid molecules of the same type aggregate spontaneously within the bilayer to form crystalline patches when their individual T_m are reached. In biological membranes, saturated and unsaturated fatty acid chains are usually bonded together in the lipid molecule, so that phase separations of this kind may not occur under physiological conditions.

Another determinant of membrane fluidity is cholesterol. Above T_m cholesterol decreases membrane fluidity. Below T_m, cholesterol increases membrane fluidity. At high concentrations found in most eukaryotic plasma membranes it also prevents the hydrocarbon chains from coming together and crystallizing and prevents possible phase
separation. In addition to affecting fluidity, cholesterol decreases the permeability of lipid bilayers to small water-soluble molecules and is thought to enhance both the flexibility and the mechanical stability of the bilayer (Chapman & Benga, 1984).

1.1.3 Membrane lipid composition

The plasma membranes of many eukaryotic cells contains not only large amounts of cholesterol but also a variety of phospholipids. The plasma membrane of many mammalian cells, for example, contains several major phospholipids: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) and sphingomyelin (SPH). The structure of PC, PE, PS and PI are shown in Figure 1.1.

![Figure 1.1 The structure of PC, PE, PS and PI.](image)

At physiological pH only PS and PI carry a net negative charge, the other three are electrically neutral zwitterions, carrying one positive and one negative charge. The lipid compositions of several biological membranes are compared in Table 1.1. Membrane lipids constitute a two-dimensional solvent for proteins in the membrane, and it may be that some membrane proteins can function only in the presence of specific
Approximate Lipid Compositions of Different Cell Membranes

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Liver Plasma Membrane</th>
<th>Erythrocyte Plasma Membrane</th>
<th>Myelin</th>
<th>Mitochondrion (inner and outer membranes)</th>
<th>Endoplasmic Reticulum</th>
<th>E. coli</th>
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Table 1.1 The lipid compositions of different cell membranes (from Alberts et al., 1989).

phospholipid headgroups. This may be why the eukaryotic plasma membrane contains such a variety of phospholipids, with headgroups that differ in size, shape, and charge.

1.1.4 Lipid asymmetry

The lipid compositions of the two halves of the lipid bilayer in those membranes that have been analyzed are strikingly different. The outer leaflet is generally rich in the lipid molecules that have choline in their headgroup such as PC and sphingomyelin, whereas the phospholipid molecules that contain a terminal primary amino group such as PE and in particular PS, preferentially occupy the inner leaflet. In the human red blood cell membrane, 82% of sphingomyelin and 76% of the PC is located in the outer leaflet (Verkleij et al., 1973), while 80% of the PE and virtually all of the PS is in the inner leaflet (Verkleij et al., 1973; Gordesky et al., 1975; Zwaal et al., 1975).

Figure 1.2 shows the asymmetric distribution of phospholipids between inner and outer
Figure 1.2 Asymmetric distribution of Phospholipids between inner and outer layer of human red cell membranes (A) and pig platelet surface membranes (B). TBL, total phospholipid. Adapted from Zwaal (1978).

Layer of human red cell membranes (A) and pig platelet surface membranes (B) (Zwaal, 1978). The nonrandom distribution of lipids is not absolute and varies, to some extent, with cell types. The acyl chains of PC and sphingomyelin are more saturated than those of PE and PS; therefore, the asymmetry in the distribution of hydrocarbon tails makes the inner monolayer more fluid than the outer layer.

Two mechanisms have been proposed as responsible for maintaining the asymmetric distribution of aminophospholipids in red blood cells. Firstly, Haest (1982) proposed that cytoskeletal proteins contribute to the maintenance of the inside
orientation of the aminophospholipids by selective interactions that do not occur with the cholinephospholipids. Secondly, Seigneur et and Devaux (1984) have reported the existence of an energy-dependent transport process that specially moves aminophospholipids from the outer to inner leaflet of cell membrane.

Studies with liposomes and monolayer lipid films have demonstrated that the major cytoskeletal component, spectrin (Haest, 1982; Haest et al., 1977; Mombers et al., 1979; Cohen et al., 1986; Sikorski et al., 1987), and band 4.1 (Sato & Ohnishi, 1983; Cohen et al., 1988; Shiffer et al., 1988; Rybicki et al., 1988) specially interact with PS. These data suggested that both spectrin and band 4.1 contribute to the maintenance of phospholipid asymmetry by their capacity to ‘fix’ PS to the inner leaflet. However, this conclusion has been questioned, and the role of cytoskeleton-PS interactions in the maintenance of membrane lipid asymmetry has not been fully defined.

When spin-labelled PS and PE were added to red blood cells, it was shown that PS and PE are selectively transported from the outer to the inner leaflet of erythrocytes, providing the cells contained hydrolysable MgATP (~1 mM) (Seigneur et Devaux, 1984). The half-times of outside-inside movement of PS and PE analogues were recorded as 5 and 60 min, respectively, at 37°C. This movement is considerably faster than that expected from passive diffusion. Similar observations have been obtained with short-chain lipids (Daleke & Huestis, 1985), long chain radioactive lipids (Tilley et al., 1986), or fluorescence lipids (Connor & Schroit, 1987) in red blood cells. These findings indicate that lipid transport in cell membranes is mediated by a facilitated transport mechanism involving lipid-specific transporters. Although absolute rates of transport are influenced by the length of the fatty acid side-chains and the presence of
a particular reporter group, translocation rates are primarily determined by the chemical nature of the polar head group.

Recent studies have indicated that a component of erythrocyte membrane band 7 (polypeptides in the 30 kDa to 32 kDa region) might be involved in the transmembrane movement of PS (Schroit et al., 1987; Connor & Schroit, 1988). This polypeptide was recently identified as part of a set of closely related isoforms that associated with the Rh blood group system, since both substrate- and inhibitor labelled polypeptides were specifically immunoprecipitated with monoclonal antibodies directed against the Rh antigen (Schroit et al., 1990). A component of the Rh blood group system therefore seems likely to be involved in the maintenance of membrane lipid asymmetry. The Rh protein is a multispansing membrane polypeptide (Cherif-Zahar et al., 1990; Avent et al., 1990), which is common to other membrane proteins associated with transport and channel functions. However, the polypeptide involved in lipid movement is not dependent on Rh antigenicity.

Devaux and colleagues, on the other hand, proposed that the aminophospholipid translocase is a Mg\(^{2+}\)-ATPase with a molecular weight in the range of 115 to 150 kDa (Zachowski et al., 1889; Zachowski & Devaux, 1990; Morrot et al., 1990), but they by no means prove this protein to be the translocase. Since the transmembrane movement of PS requires hydrolysable ATP, and sequence analysis indicates that the Rh protein does not contain ATP binding sites (Avent et al., 1990), it is reasonable to assume that the function of the translocase would depend on the activity of a distinct ATP-utilizing enzyme. Thus, observations suggesting the involvement of both Rh protein and Mg\(^{2+}\)-ATPases in PS transport are not mutually exclusive. The transport of fluorescent labelled PS analogues was shown to require the coordinated and
complimentary participation of a 32 kDa polypeptide and a presumably distinct protein located at the endofacial membrane surface (Connor & Schroit, 1990). Whether this protein is a Mg$^{2+}$-ATPase, protein kinase, or other unidentified protein remains to be determined.

Data from several laboratories indicate that an active aminophospholipid translocase is not required to maintain asymmetry once it has been established, but the participation of the cytoskeleton in this process is not excluded (Haest, 1982; Tilley et al., 1986; Comfurius et al., 1990; Connor & Schroit, 1990; Henseleit et al., 1990; Vidal et al., 1989). The actual role of the translocase could lie in its ability to regenerate phospholipid asymmetry once it has been disturbed.

Lipid bilayer membranes that contain anionic phospholipids are known to be procoagulant (Rosing et al., 1980; Jackson & Nemerson, 1980), the most potent of these lipids being PS (Rosing et al., 1988). The main function of the membrane is to provide a catalytic surface on which coagulant factors interact, thereby increasing their local concentrations. Two consecutive reactions are greatly accelerated in the presence of lipid surfaces containing phosphatidylserine (Figure 1.3). The first is the conversion of factor X into an active serine proteinase, factor Xa. This reaction is catalyzed by an active proteinase, factor IXa, in conjunction with a nonenzymatic protein, factor VIII, in the presence of calcium. Once factor Xa is formed, it assembles together with another nonenzymatic protein, factor V, and calcium on the anionic phospholipid surface. This prothrombinase complex efficiently converts prothrombin into thrombin. The combined decrease in the $K_m$ of the two reactions caused by a negatively charged phospholipid surface produces more than a million-fold increase in the rate of thrombin formation. This enables that the assays of procoagulant activity of cell and liposome
Figure 1.3 Assembly of the tenase- and prothrombinase complex on the platelet plasma membrane (from Schroit & Zwaal, 1991).

surface to provide a sensitive and reliable means of detecting surface exposure anionic phospholipids, especially phosphatidylserine.

1.1.5 Freeze-fracture electron microscopy

Freeze-fracture electron microscopy is a technique in which cells or membrane samples are frozen in liquid nitrogen and the resulting block of ice is fractured with a knife. The fracture plane tends to pass through the hydrophobic middle of membrane lipid bilayers, separating them into their two monolayers. The exposed fracture faces are then shadowed with platinum, and the resulting platinum replica is examined with an electron microscope. Two different hydrophobic fracture faces are exposed and replicated in this technique. The faces representing the cytoplasmic (or protoplasmic) half of the bilayer is called the P face. The face representing the external half of the
bilayer is called the *E* face. Band 3 proteins can be seen as distinct intramembrane particles by this technique. The technique of freeze-fracture electron microscopy is widely used to investigate the polymorphism and deformations in lipid membranes.

### 1.2 Protein

The working molecules of the cell are proteins. They determine the shape and structure of the cells, control the permeability of membranes, regulate the concentrations of required metabolites, serve as the main instruments of molecular recognition and catalysis, control the functioning of genes etc. These incredibly diverse tasks are performed by molecules synthesized from only 20 different amino acids. All of the 20 amino acids have in common a central carbon atom (C*) to which are attached a hydrogen atom, an amino group (NH$_2$), and a carboxyl group (COOH). What distinguishes one amino acid from another is the nature of the side chain attached to the C$_\alpha$ atom. There are 20 different side chains specified by the genetic code; others occur, in rare cases, as the products of enzymatic modifications after translation.

In proteins, amino acids are joined by peptide bonds. Proteins are polypeptide chains. The amino group of the first amino acid of a polypeptide chain and the carboxyl group of the last amino acid remain intact, and the chain is defined as running from its amino terminus to carboxyl terminus. The formation of a succession of peptide bonds generates a "main chain" or "backbone" from which project the various side chains. The basic repeating unit along the main chain is (NH-C$_\alpha$H-CO). Depending on the chemical nature of the side chain, the amino acids are usually divided into three classes. The first class comprises those with strictly hydrophobic side chains Ala (A), Val (V), Leu (L), Ile (I), Phe (F), Pro (P), and Met (M). The second class comprises those with charged side chains Asp (D), Glu (E), Lys (K), and Arg (R). Those with polar side
chains form the third class: Ser (S), Thr (T), Cys (C), Asn (N), Gln (Q), His (H), Tyr (Y), and Trp (W). The amino acid glycine (G), which only has a hydrogen atom as a side chain, has special properties and usually considered either as a fourth class or belonging to the first class.

1.2.1 Primary, secondary, tertiary, quaternary structure

Protein molecules are organised in a structural hierarchy. The amino acid sequence of a protein’s polypeptide chains is called its primary structure. This is the most fundamental description of a protein, and defines all of its properties. Different regions of the sequence form local regular secondary structures, such as α helices or β strands. The tertiary structure is formed by packing such structural elements into one or several compact globular units called domains. The final protein may contain several polypeptide chains arranged in a quaternary structure.

1.2.1.1 α-helix

The α-helix is an important element of secondary structure. Hydrogen bonds are formed between the carbonyl oxygen of residue \( i \) and the NH of the \( i + 4 \) residue alone the peptide chain. The hydrogen bonds all point in the same direction and are nearly parallel to the helix axis. Different side chains have been found to have weak but definite preferences either for or against being in α helices. Thus Ala (A), Leu (L), and Met (M) are good α helix formers, while Pro (P), Gly (G) are very poor α helix formers (O’Neil & DeGrado, 1990).

1.2.1.2 β-sheet

The second major structural element found in proteins is the β sheet. β sheet is formed by β strands aligned adjacent to each other. The hydrogen bonds form between CO groups of one β strand and NH groups on an adjacent β strand. The β sheets that
are formed from several such β strands are "pleated" with Cα atoms successively projected slightly above and below the plane of the β sheet. The side chains follow this pattern being projected alternatively above and below the β sheet. β sheets can have their β strands either parallel or antiparallel.

1.2.1.3 Turns

Reverse turns are structural features of polypeptides and proteins that involve four (for the β-turn) or three (for the γ-turn) successive amino acid residues, and are characterized by an intramolecular hydrogen bond that produces a reversal of the direction of the polypeptide chain by ~180°. For β-turns, the carbonyl oxygen of i unit is hydrogen bonded to the NH of i +3 residue (Fig. 1.4). Three types of β-turns were predicted by Venkatachalam (1968) on the consideration of steric hindrance. Data from crystal-structure studies of more than 100 globular proteins have revealed that a
substantial portion of the amino acid residues in globular proteins occur in β turns (Bernstein et al., 1977). The γ-turn is formed by three amino acid residues, i, i+1, and i+2, and is characterised by the presence of two hydrogen bonds. Three different energetically stable conformations have been proposed.

Reverse turns have the property of making an extended polypeptide chain compact. This compactness is implicated in the specific functions that a given protein carries out. A substantial portion of surface residues, or those exposed to the solvent where most of the enzymatic reactions take place, are in reverse turns. Compared to the frequent occurrence of β turns in proteins and peptides, only 10γ turns have been identified in all globular protein structures known to date (Baker and Hubbard, 1984).

1.2.2 Membrane proteins

Although the basic structure of biological membrane is provided by the lipid bilayer, most of the specific functions are carried out by membrane proteins. Because lipid molecules are small in comparison to protein molecules, there are always many more lipid molecules than protein molecules in membrane - approximately 50 lipid molecules for each protein molecule in a membrane that contains 50% protein by mass.

The polypeptide chain of many membrane proteins crosses the lipid bilayer one or more times. These transmembrane proteins are amphipathic. They have hydrophobic regions that pass through the membrane and are in contact with the hydrophobic tails of the lipid molecules in the interior of the bilayer, and hydrophilic regions that are exposed to water on both sides of the membrane. The hydrophobicity of some of these membrane proteins is increased by the covalent attachment of a fatty acid chain which is inserted in the cytoplasmic leaflet of the bilayer. Some intracellular membrane proteins are associated with the bilayer solely by means of such a fatty acid chain, while
some cell-surface proteins are attached to the bilayer only by a covalent linkage to phosphatidylinositol (PI), a minor phospholipid, in the outer lipid monolayer of the plasma membrane.

Other proteins associated with membranes do not extend into the hydrophobic interior of the lipid bilayer but are bound to one or other face of the membrane by noncovalent interactions with other membrane proteins. Many of these can be released from the membrane by relatively gentle extraction procedures, such as exposure to solutions of high or low ionic strength or by altering pH, thus interfering with the protein-protein interactions but leave the lipid bilayer intact. These proteins are referred as **peripheral membrane proteins**. By contrast, transmembrane proteins, proteins linked to phosphatidylinositol, and some proteins held in the bilayer by a fatty acid chain, as well as some other tightly bound proteins, can be released only by disrupting the bilayer with detergents or organic solvents, are called **integral membrane proteins**.

When the plasma membrane proteins of the human red blood cell are studied by SDS polyacrylamide-gel electrophoresis, approximately 15 major protein bands are detected, varying in molecular weight from 15 kDa to 250 kDa. Three of these proteins: spectrin, glycophorin, and band 3, account for more than 60% (by weight) of the total membrane protein. Each of these proteins is arranged in the membrane in a different manner (Steck, 1974).

Glycophorin is a small transmembrane glycoprotein (131 amino acid residues) with most of its mass on the external surface of the membrane. Its structure is representative of a common class of transmembrane glycoproteins that traverse the lipid bilayer as a single α-helix: so called single pass membrane proteins.

Band 3 protein is a multipass membrane protein, traversing the membrane in a
highly folded conformation. Each red blood cell contains about $10^6$ band 3 polypeptide chains, which are thought to form dimers and possibly tetramers in the membrane. Band 3 protein has been identified as an anion transport protein which ejects $\text{HCO}_3^-$ in exchange for $\text{Cl}^-$ when red cells move through the lungs to dispose of the $\text{CO}_2$ they accumulate in the tissues.

Most of the protein molecules associated with the human red blood cell membrane are peripheral membrane proteins associated with the cytoplasmic side of the lipid bilayer. The most abundant of these proteins is spectrin, a long thin flexible rod about 100 nm in length that constitutes about 25% of the membrane-associated protein mass (about $2 \times 10^5$ copies per cell). It is composed of two very large polypeptide chains, $\alpha$ spectrin (~240 kDa) and $\beta$ spectrin (~220 kDa). The spectrin heterodimers associate head to head to form tetramers that are linked together into a network by junctional complexes composed of short actin filaments and band 4.1 (and two or three other proteins). This cytoskeleton is linked to the membrane by the indirect binding of spectrin tetramers to band 3 protein via a large intracellular attachment protein called ankyrin, and may also be linked by the binding of 4.1 proteins to glycophorin. It is this spectrin-based cytoskeleton that underlines the red blood cell membrane, maintaining the structural integrity and biconcave shape of this membrane.

Membrane proteins do not flip-flop across the bilayer, and they rotate about an axis perpendicular to the plane of the bilayer (rotational diffusion). In addition, many membrane proteins are able to move laterally in the membrane (lateral diffusion). On the other hand, some cells have ways of immobilizing specific membrane proteins and of confining both membrane protein and lipid molecules to particular domains in a continuous lipid bilayer.

1.3 Osmotic balance of cells

1.3.1 Membrane transport of small molecules

Because of its hydrophobic interior, the lipid bilayer is a highly impermeable barrier to most polar molecules. The rate at which a molecule diffuses across such a lipid bilayer, depends mainly on the size of the molecule and its relative solubility in oil. Small and/or hydrophobic (or nonpolar) molecule will generally diffuse across a bilayer rapidly. Figure 1.5 shows the relative permeability of a synthetic lipid bilayer to different classes of molecules.

Water (18 Da) diffuses very rapidly across lipid bilayers. Lipid bilayers are highly impermeable to all charged molecules (ions), no matter how small they are.

Figure 1.5 The relative permeability of a synthetic lipid bilayer to different classes of molecules (from Alberts et al., 1989).

In order to transport water-soluble molecules into or out of cells, plasma membranes contain various transport proteins, each of which is responsible for
Comparison of Ion Concentrations Inside and Outside a Typical Mammalian Cell

<table>
<thead>
<tr>
<th>Component</th>
<th>Intracellular Concentration (mM)</th>
<th>Extracellular Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na⁺</td>
<td>5-15</td>
<td>145</td>
</tr>
<tr>
<td>K⁺</td>
<td>140</td>
<td>5</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>0.5</td>
<td>1-2</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>10⁻⁴</td>
<td>1-2</td>
</tr>
<tr>
<td>H⁺</td>
<td>8 × 10⁻⁵ (10⁻⁷ M or pH 7.1)</td>
<td>4 × 10⁻⁵ (10⁻⁷ M or pH 7.4)</td>
</tr>
<tr>
<td>Anions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cl⁻</td>
<td>5-15</td>
<td>110</td>
</tr>
</tbody>
</table>

Table 1.2 Comparison of ion concentrations inside and outside a typical mammalian cell (from Alberts et al., 1989).

Transferring a specific molecule or a group of closely related molecules across the membrane. A combination of selective permeability and active transport across the plasma membrane creates large differences in the ionic composition of the cytosol compared with the extracellular fluid (Table 1.2). This enables cell membranes to store potential energy in the form of ion gradients.

1.3.2 Osmotic balance of cells

The osmolarity of the extracellular fluid is usually due mainly to small inorganic ions. The charged macromolecules and metabolites inside the cell attract small inorganic ions due to the Donnan effect. Animal cells control their intracellular osmolarity by actively pumping out inorganic ions, such as Na⁺, so that their cytoplasm contains a lower total concentration of inorganic ions than the extracellular fluid, thereby compensating for their excess of organic solutes.

When the osmolarity (tonicity) of the extracellular fluid of human red blood cells
is changed, water will move into or out of cells down its concentration gradient, a process called \textit{osmosis}. If cells are placed in a hypotonic solution, there will be a net movement of water into the cells, causing them to swell and lyse. Conversely, if cells are placed in a hypertonic solution, they will shrink.

\textbf{1.4 Membrane fusion}

Membrane fusion is one of the essential events involved in many biological cellular processes. Some membrane fusion processes are exoplasmic: the exoplasmic (outer) leaflets of the fusing bilayers make initial contact. Such exoplasmic fusion events include virus-cell fusion, myoblast fusion, sperm-egg fusion etc. The fusion processes in contrast to exoplasmic fusion are endoplasmic fusion in which the endoplasmic (cytoplasmic) leaflets make initial contact. Such endoplasmic fusion events include regulated exocytosis, endosome-endosome fusion, fusion of intracellular transport vesicles: endoplasmic reticulum to Golgi and inter-Golgi fusion (White & Blobel, 1989).

Cell fusion can be also induced artificially by chemical fusogens such as PEG (polyethylene glycol), and by physical means such as high intensity electric field pulses (electrofusion) to produce hybridoma cells. Liposomes have been widely used as a model system to study the molecular mechanism of membrane fusion and used to cellular delivery of the liposome contents.

There are many possible molecular pathways for various membrane fusion events and also biological membranes have very complex nature. However, there are still some common steps in various membrane fusion processes:

1. adhesion or aggregation between two apposing membranes,
2. membrane mixing,
3. cytoplasmic content mixing,

4. enlargement of the fusion pore.

It is suggested for a membrane fusion process, the hydration force of membranes need to be overcome to enable membranes to be fused (Rand, 1981).

Water is indispensable for maintenance of structural and functional integrity of biological membranes and macromolecules. It is the amphipathic nature of the lipid molecules that causes them to form bilayers spontaneously in aqueous environment. When amphipathic molecules are surrounded on all sides by an aqueous environment, they intend to aggregate so that their hydrophobic tails are buried in the interior and their hydrophilic heads are exposed to water.

When water is added to dry phospholipids, the adjacent bilayers swell, and water is incorporated between the bilayers. The amount of swelling depends upon the nature of the polar head group. In addition to inserting between bilayers, a significant fraction of the added water binds to the phospholipids. This fraction of the water is known as "bound" water (Hauser, 1975a,b).

It is suggested that there is a hydration shell around the headgroup of phospholipids, and the nature of the water in this hydration shell has been studied by various techniques (Crowe & Crowe, 1984). It has been found that for different phospholipids, the amounts of bound water are different (Rand & Parsegian, 1988). The water molecules in the hydration shell are considered to be in dynamic exchange with water molecules in bulk phase aqueous medium. The hydration state of phospholipids in bilayers is suggested to affect the nature of lipid-lipid interactions (Crowe & Crowe, 1984).

Membrane close apposition is a prerequisite for membrane fusion. The forces
that membrane experience on mutual approach have been measured for a wide variety of phospholipid bilayers in multilamellar arrays (Rand, 1981). These physical forces are long-range Van der Waals attraction, electrostatic repulsion for bilayers that carry a net surface charge, and hydration repulsion. The hydration force is supposed to be due to the existence of a hydration shell at the surface of lipid bilayer surface. When the separations between membranes are smaller than 20-30 Å (where contact and fusion begin), all bilayers experience a strong repulsive force that grows exponentially with a characteristic distance of 2-3 Å (Rand, 1981). The origin of the hydration force is the cost of dehydrating hydrophilic groups. This repulsive force is the major barrier to membrane contact.

Acidic phospholipids, especially PS liposome fusions induced by divalent or polyvalent cations have been well studied (Ohki, 1987; Düzgüneş et al., 1987). It is suggested that the increase of hydrophobicity at the surface of the membrane after the interaction of divalent or polyvalent cations with the headgroup of acidic phospholipid play a role in the fusion of acidic phospholipid membranes (Ohki, S., 1982; Ohki, 1987; Düzgüneş et al., 1987). It has been proposed that the effect of Ca$^{2+}$ is to induce a close approach of two apposing PS vesicles by displacement of water from the interbilayer space and the formation of an anhydrous, trans, PS-Ca$^{2+}$ complex spanning the interbilayer space (Portis et al., 1979). FTIR studies of the effect of calcium ions on phosphatidylserine (PS) has shown that Ca$^{2+}$ binds to the PS phosphate as a bidentate ligand and causes a dehydration of the phosphate ester (Dluhy, RA et al., 1983).

Polyethyleneglycol (PEG) is a widely used chemical fusogen and can be used to induce the fusion between cells (Ahkong et al., 1975) and liposomes (Herrmann et al., 1983; Burgess, S. W., 1992). It is suggested that the dehydration of membrane
surfaces by PEG play a role in membrane fusion induced by PEG. However, Ahkong and Lucy (1986) proposed that, while the dehydration of PEG-treated cells brings them into close contact, it is their rehydration that causes cell fusion.

Although extensive investigations on membrane fusion have been done to understand the mechanism of membrane fusion, and the possible role of dehydration in membrane fusion process, its molecular mechanism is not yet understood, especially for physiological occurring cellular and intracellular fusion, and protein-mediated neutral phospholipid liposome fusion. Electrofusion has been used to investigate the mechanism of cellular fusion. More work needs to be done to understand the molecular mechanism of these fusion events, and how they overcome hydration repulsion force of membranes although their molecular pathways are different.

In this thesis, work has been concentrated on the study of the mechanism of membrane fusion in electrically-induced erythrocyte fusion and the mechanism of membrane fusion induced by influenza and HIV fusogenic viral peptides.
Chapter Two

Membrane fusion without cytoplasmic fusion (hemi-fusion) in erythrocytes that are subjected to electrical breakdown

2.1 Introduction

2.1.1 Electrofusion

Electrofusion is a method in which cells are induced to fuse by the pulsed electric field. The phenomenon of electrofusion is closely related to that of electroporation (electropermeabilization, electrical breakdown). There are several major steps in a process of electrofusion: 1. cell contact, 2. electroporation, 3. cell fusion.

Cell contact can be established by mechanical manipulation (including the tight contact of cells in an overgrowing tissue culture plate), chemical treatment, dielectrophoresis and other means. Among them, the application of the phenomenon of dielectrophoresis to facilitate cell contact by Zimmermann’s group has made the electrofusion method more widely useful.

2.1.1.1 Dielectrophoresis

The movement of particles under the influence of a nonuniform AC (alternating current) field has been termed dielectrophoresis (Pohl, 1978). This phenomenon can be demonstrated for any polarizable object, charged or not.

When placed in an electric field, polarizable bodies become polarized as a result of the induction and reorientation of dipoles. In a uniform electrical field, a polarized body will not experience a net translational force. In nonuniform fields, the charge in the stronger field experiences a stronger force than the charge at the opposite side of the polarized object, so the object tends move towards the region of higher field
strength. This movement occurs even in AC fields because the induced dipoles reorient in phase with the changing polarity of the electrodes. Dielectrophoresis is in contrast to electrophoresis, which involves the movement of charged bodies in DC (direct current) fields. Electrophoresis does not occur in AC field.

In AC fields, the cells create local distortions in the electric field, causing the ends of each cell to act as tiny electrodes. Consequently, when cells undergoing dielectrophoresis approach each other, they become mutually attracted and finally line up in rows, parallel to the lines of electric field. These chains are called pearl chains.

The physical basis of dielectrophoresis lies in the difference between the permittivities of the cell (or particle) and its surrounding medium and described by Pohl (1978), Pething (1979), and Arnold & Zimmermann (1984).

For dielectrophoresis of cells in electrofusion, several aspects are relevant: 1. dielectrophoresis and cell alignment are frequency dependent; 2. the rate of cell alignment depends on both the magnitude of the electric field and the radius of the cell (the smaller the cell, the larger the electric field that must be applied to achieve alignment); 3. cell alignment is achieved most easily in media of low ionic strength; 4. compared to DC field for electroporation, AC field for dielectrophoresis is a lower intensity electric field. More details are given by Bates et al. (1987).

2.1.1.2 Electroporation

Electroporation (electropermeabilization, electrical breakdown) is a phenomenon in which the membrane of a cell exposed to high-intensity electric field pulses undergoes physical breakdown and is temporarily destabilized in specific regions of the cell when the electric field pulses drive the membrane potential to a critical voltage ($V_c$). During the destabilization period, the cell membrane is highly permeable to
exogenous molecules present in the surrounding media.

Figure 2.1 The most commonly used waveforms of DC pulses. (A) Exponential decay pulse, which is generated by discharging a capacitor. (B) Rectangular pulse (or square pulse).

In both of electroporation and electrofusion, a pulsed electric field is always used to induce membrane breakdown in the targeted cells to prevent irreversible cell damage. Conventional electroporation and electrofusion methods typically use a DC (direct current) electric field in the form of either a rectangular (square) or an exponential decay pulse (Figure 2.1A and 2.1B).

When a spherical cell is exposed to an electric field, a membrane potential is induced, which is given by Arnold & Zimmermann (1984):

\[ V_m = 1.5rE \cos \theta \]  

(E.2.1)

Where \( V_m \) is the induced membrane potential, \( r \) is the cell radius, \( E \) is the electric field strength, and \( \theta \) is the angle between the normal vector of the membrane and the electric field. From this equation, several points can be seen: 1. The induced membrane potential is proportional to the strength of the applied field. 2. At a given field strength the induced membrane voltage is directly proportional to the radius of the cell. 3. The
Figure 2.2 Effect of an applied electric field on the extent of electroporation and the distribution of field-induced pores. (a) \( V_m \) equals the cell breakdown potential \((V_c)\), and pores form at the poles. (b) The larger applied electric field induces electroporation over more of the cell surface.

induced membrane potential is not uniform, the membrane voltage is maximum at cell poles (where the direction of the overall electric field direction (vector) crosses the membrane perpendicularly, \( \theta = 0 \) in Fig.2.2a) and is decreased towards the cell equator.

The critical voltage \( V_c \) for electroporation has been recorded for a wide range of animal and plant cells, as well as for liposomes (Teissie and Tsong, 1981). \( V_c \) ranges from 0.5 to 1.5 V depending on the particular system and precise experimental conditions. The value of \( V_c \) can vary depending on a number of parameters, including temperature, membrane composition, and the pulse width. There is a compensating relationship between the field strength and the pulse width. It has been observed that
when an electric pulse of very narrow pulse width is used, the field strength required to give effective poration is usually quite high. Conversely, when the pulse width is large, the required field strength is much lower (Chu et al., 1987).

When $V_c$ is reached first at the cell poles, increasing the pulse length or voltage should result in poration over a large portion of the cell surface (Fig. 2.2b). The size of the pores could be controlled. A higher intensity, a longer pulse width, or a lower ionic strength of the suspending medium all lead to formation of larger pore sizes.

Why are membranes exposed to the high-intensity electric field pulses electropermeabilized? Coster and Zimmermann (1975) proposed that the applied electric field exerts enough force on the membrane to compress it physically. Ordinarily, this compression is balanced by the membrane's elastic restoring forces. If compressed sufficiently, however, the restoring force is overwhelmed and the membrane collapses. If the membrane voltage reached during the short pulse is not excessive, the membrane breakdown will be reversible. There is considerable theoretical justification for this model (Crowley, 1973; White & Chang, 1981; Dimitrov & Jain, 1984). Many other model mechanisms have also been proposed to explain pore formation (Abidor et al., 1979; Berg et al., 1979; Berg et al., 1984; Tsong, 1990).

Chang and Reese (1990) provided the first morphological evidence that the electric field induced permeabilization of cell membrane is related to the formation of transient membrane pores by using rapid-freezing freeze-fracture and freeze-substitution electron microscopy techniques.

Their observations show that the evolution of electropores is a dynamic process, which may be divided roughly into three stages: In the first stage (consisting of the first few milliseconds after the electrical pulsing), pores were created. Initially, the newly
formed pores were very small (< 2 nm); within a few millisecond, they expanded rapidly to a diameter of 20-40 nm.

In the second stage (from a few milliseconds to several seconds), the pore structures became relatively stable. After pores had expanded to 20-120 nm in the first 20 ms of this stage, they remained more or less unchanged during the next few seconds.

In the third stage (from seconds to minutes), pores underwent a resealing process. The pore diameters became reduced with time. However, some of the partially resealed pores might have a long lifetime. The diameters of the transient electropores as large as 20-120 nm should be sufficient to allow most macromolecules to pass through. The life time of these membrane pores (in the range of seconds) was also long enough to allow elongated molecules (such as DNA) to diffuse in the cell (Chang, 1992).

The reversibility of pore formation is an important factor in determining the viability of electrically fused and permeabilized cells. Membrane break down is reversible, provided that the field strength and pulse duration are not excessive. The rate of resealing depends on size of pores, temperature, cell type or membrane composition. At 4°C, the pores will remain open for long time (hours or days). At 37°C, the resealing is complete within minutes to hours (Kinosita & Tsong, 1977b; Zimmermann et al., 1980; Teissie & Tsong, 1981). Resealing of pure lipid vesicles (Teissie & Tsong, 1981) is orders of magnitude faster than for red blood cells (Zimmermann et al., 1980; Serpersu et al., 1985) and plant protoplasts (Steinbiss, 1978). Membrane proteins probably stabilize the pores and slow the resealing process.

Chang (1989) suggested that an electropore could be formed by an irreversible breakdown of a localized patch of cell membrane, which is enclosed within the holes
of the cytoskeletal network. The predictions derived from the hypothesis are in good agreement with the experimental findings by Chang and Reese (1990). Not only does the dimension of the volcano-shape membrane openings match the size of the holes in the cytoskeletal network, the dynamics of pore formation also indicate that the membrane openings are stabilized for a few seconds when they reach a size comparable to that of the network holes, before the pore diameters become reduced with time. These results suggest that membrane-cytoskeletal interactions may play an important role in the shaping of electropores (Chang, 1992).

It was suggested by Chang (1992) that the membrane pores are not only shaped by the primary effects of interactions between the applied electric field and the cell membrane, but also by a secondary effect of material flow following the initial permeabilization of the cell membrane induced by the electric field. The diameters of their observed membrane openings were larger than those predicted in many theories that consider principally the primary effects (Chernomordik et al., 1983; Powell & Weaver, 1986). This difference suggests that material flow could be important in shaping the pores. Chang and Reese (1990) found in their study that the membrane opening always appeared as a volcano pointing outward from the cell, regardless of whether the membrane was facing the anode or the cathode electrodes. This observation is consistent with the view that formation of pores may be influenced by an ejection of cellular contents. They thought that the shape and direction of opening cannot be explained by water movement because the same phenomenon was observed when the pulsing medium was hypo-osmotic. They suggested that one possible cause of such outward-pointing pores is the flow of haemoglobin. The results of their freeze-substitution study suggest a partial loss of haemoglobin near the membrane pores.
Kinosita and Tsong (1977a) studied the erythrocytes in 150 mM NaCl, 7 mM phosphate buffer, pH7.0 or the mixture of this NaCl solution with 272 mM sucrose solution at different ratio (both solutions are isotonic). When the cells were treated with a single 3.7 kV/cm electric pulse (square wave, 20 µs), K⁺ ions leaked out within a few seconds. Likewise, external Na⁺ entered rapidly. In the isotonic saline solution, the pores are small, they allow passage of K⁺ and Na⁺ but not of sucrose and haemoglobin molecules. The pores are larger in low ionic conditions and permit permeation of sucrose molecules, but under no circumstances can haemoglobin leak out as the direct result of the electric field pulse. The contradictory results of Kinosita & Tsong (1977a) and Chang & Reese (1990) on whether haemoglobin leaks from electropermeabilized erythrocytes are probably due to the different size of pores resulted from the different pulsation conditions they used.

Tsong (1989, 1990) proposed that after the cell membranes were exposed to electric field pulses, the membranes lost their function as a semipermeable barrier, the term "isotonic" loses its meaning for a solution of saline, sucrose, or a mixture of saline and sucrose. However, the membranes are still impermeable to the cytoplasmic macromolecules. The cell begin to swell in such an "isotonic" suspension in the time scale of seconds to min owing to the osmotic pressure of these cytoplasmic macromolecular contents (which have an equivalent pressure of 20-30 mOs). Paralleling the cell swelling, there is also a gradual shrinking of the diameter of the electropores. The colloid osmotic swelling eventually leads to the rupture of the cell membranes and hence the lysis of the cells when the cell volume approaches 155% of the normal volume (Kinosita & Tsong, 1977a; Tsong, 1983).
The swelling and the consequent haemolysis of the field treated red cells could be prevented by adding molecules larger than the sizes of the electropores to the external medium to balance the colloidal osmotic pressure of the cytoplasmic fluid. Oligosaccharides and small proteins have been used (Kinosita & Tsong, 1977b; Tsong, 1983).

Drugs, which are impermeable to cell membranes and have a small molecular weight, e.g. less than 1000, could be readily loaded into human erythrocytes. For cells with fragile membranes, the use of different compositions of pulsing medium and resealing medium has been recommended (Tsong & Kinosita, 1985).

### 2.1.1.3 Electrically induced cell fusion and its molecular mechanism

It was first found that electrofusion can occur when an electric field pulse is applied while two cells are in contact (Zimmermann, 1982; Teissié et al., 1982). Later Sowers (1986) found that erythrocyte fusion occurred nearly as efficient whether the DC field pulses were given before or after cell contacts were established. Even a 120 seconds interval between application of the DC field pulses and the establishment of membrane-membrane contacts did not significantly reduce fusion. This observation has been confirmed by Teissié and Rols (1986) for mammalian cells and recently extended to plant protoplasts (Montané et al., 1990) and indicates that there is a long-lived fusogenic state in electroporated cell membranes.

In a pearl chain of cells formed along the electric field direction, hydration repulsion forces between the cell membranes at the contact sites may be partially overcome by the tight contact induced by dielectrophoresis. DC field pulses preferentially induce electroporation around the contact sites when the induced membrane potential $V > V_c$, the critical voltage. Membrane fusion may occur when
the two opposed fusogenic membranes induced by electroporation are brought together by dielectrophoresis. If the fusogenic state in the electroporated cell membrane is long-lived, then membrane fusion can occur when electroporated cell membranes are brought together sometimes after the DC field pulses are applied. This fusogenic state in the electroporated cell membranes was reported to be very highly localized and the locations of these fusogenic areas were observed to coincide with the pole area of the electroporated cells. It was found that the fusogenic area laterally diffuses very slowly or not at all (Sowers, 1986, 1987). The fusion yields of electroporated cells brought together some time later after the application of electric field pulses showed a time-dependent decay (between the electric field pulses and the membrane-membrane contact) (Sowers, 1986, 1987). What is the molecular structure of this fusogenic state of electroporated cell membranes? This is the core question for understanding the molecular mechanism of electrically induced cell fusion. Some explanations have been suggested for the fusogenic state in the electroporated cell membranes. Tsong (1990) suggested that loss of lipid asymmetry, existence of pores, modification of lipid compositions or protein meshwork, and other unspecified changes explain fusogenic states. Teissié and Rols (1986) implied that the absence of diffusion and/or spreading of the fusogenic state in the electropermeabilized cell membrane suggest a connection to the cytoskeleton.

The phenomenon of electrically induced cell fusion seems a more complicated process than that of electroporation since there are more steps in a cellular fusion process: 1. membrane-membrane contact or aggregation, 2. membrane-membrane mixing, 3. cytoplasmic content mixing, 4. giant cell formation. Different fusion assays may only detect one stage of cell fusion. For example, the assay based on membrane
labelled probes can only detect the process of membrane mixing; the assay based on cytoplasmic labelled probes can only detect the process of cytoplasmic content mixing; the optic microscopic observation can only detect the giant cell formation.

Many factors may influence electrically induced cell fusion and many researchers have tried to find out these factors. But sometimes conclusions from different groups are contradictory. For example, Sukharev et al. (1990) showed that fibroblast electrofusion yield was proportional to the concentration of NaCl or KCl in the medium. On the other hand, studies using CHO (Chinese hamster ovary) cells (Blangero & Teissié, 1985; Rols & Teissié, 1989) showed a roughly inversely proportional relationship between fusion and ionic strength. Sowers (1989) observed that fusion yield in erythrocyte ghost has a peak with sodium phosphate buffer (pH 8.5) in the concentration range 20-30 mM and fusion yield is zero or below practical detection limits around buffer (sodium phosphate, pH 8.5) strengths of 2-5 mM.

Contradictions of these conclusions from different laboratories may be due to:
1. different cell or membrane systems are used, it has been found that cell membrane systems from different species are quite different (Sowers, 1992). 2. the ways to achieve cell contact are different. 3. different assays for fusion were used. 4. other experimental conditions such as temperature, osmolarity and composition of the solution, pulse parameters are different. 5. sometimes the instrument and sample chamber used are different.

Although the electrofusion technique has been used widely, different protocols, pulsing mediums are used in different laboratories (Hofmann, 1989). Many factors which may influence electrofusion process are still unknown. For example, why divalent ions are used in some experiments but only monovalent ions are used in other
cases? What is the role of a divalent ions? Are there any difference between divalent ions such as Ca$^{2+}$, Mg$^{2+}$? Why sucrose is used in this experiment but mannitol is used in other cases? What is the influence of temperature on electrofusion? If osmotic forces play any role in electrofusion? It is still necessary and very important to understand the molecular mechanism of electrically induced cell fusion.

### 2.1.1.4 Equipment and chambers

Homemade and commercial pulse generators are reviewed in detail by Chassay et al. (1992). Physical and practical aspects of electroporation and electrofusion, including chambers for electroporation and electrofusion are reviewed by Hofmann (1989).

### 2.1.1.5 Application of electrofusion and electroporation

Applications of electrofusion have been extended into many different areas using a wide variety of cell types. In plants, somatic hybridization of isolated protoplasts by electrofusion has been a popular method of genomic gene transfer. A second area has been hybridoma/monoclonal antibody production. The use of electrofusion techniques in this application has sometimes improved the yields and recoverability of hybridoma cells greatly in comparison to chemical fusion methods. Electrofusion is also a promising new way to study membrane fusion mechanism: it can induce fusion in high yields, and the time when the fusion process is started can be easily controlled.

Applications of electroporation have involved the introduction of both DNA and RNA to a variety of plant, animal, bacterial, and yeast cells. Other major applications are injection of drugs, proteins, metabolites, molecular probes, and antibodies for studies of cellular structure and function.

The pulsed electric field method has a number of advantages over the
conventional methods of cell permeabilization or cell fusion. Compared to other chemical or biological methods, the electric field method can produce relatively less damage to cell membranes. Electrofusion is relatively easy to perform and is faster than the traditional chemical or biological fusion techniques. The efficiency of electrofusion is generally significantly better than most alternative methods. Because the electric field method is a physical method, it can be applied to a much wider selection of cell types.

2.1.2 Fusion intermediate

One particular interest in studies of the mechanism of membrane fusion is the possible participation in the membrane merging process of intermediate structures, such as a shared bilayer (reviewed by Lucy and Ahkong (1986)). In ultrastructural investigations, an intermediate single bilayer has been found occurring in exocytosis of vascular endothelial vesicles (Palade & Bruns, 1968; Palade, 1975), zoospore secretion in Phytophthora palmivora (Pinto da Silva & Nogueira, 1977), myoblast fusion (Kalderon & Gilua, 1979), and the fusion of carrot protoplasts by poly(ethylene glycol) (Kanchanapoom & Boss, 1986). The assays on the fusion of phospholipid vesicles indicated by the diffusion of fluorescent probes have shown that, under certain conditions, an intermixing of lipid probes can occur without mixing of the vesicular contents (Ellens et al., 1985). This phenomenon has been observed by a number of investigators (Silvius et al., 1988; Düzgüneş et al., 1988), and has been termed 'hemi-fusion' (Leventis et al., 1986). Hemi-fusion may involve fusion of the outer monolayers of the vesicles into an intermediate, shared bilayer, or possibly the formation of a transient H$_{11}$ hexagonal phase as suggested by Ellens et al. (1985).

In the work on the fusion of human erythrocytes induced by poly(ethylene glycol) 6000 (Ahkong et al., 1987), it was found that carbocyanine and rhodamine
membrane probes diffused from labelled to unlabelled cells when they were dehydrated by 40% poly(ethylene glycol), but there was no corresponding movement of the cytoplasmic probe, 6-carboxyfluorescein, until the polymer solution was replaced by an isotonic buffer. The rapid diffusion of carboxyfluorescein that then occurred was thought to be consistent with the rupture by osmotic stretching of an intermediate, shared bilayer.

So far hemi-fusion in cells exposed to electrical pulses has not yet been characterised. In the present chapter, experiments are reported on the exposure to electrical breakdown pulses of human erythrocytes which were also labelled with a membrane probe and a cytoplasmic probe. The membrane probe, but not the cytoplasmic probe, diffused from labelled to unlabelled cells (i.e. hemi-fusion occurred) when the cells were attached to each other, but were not completely fused by the breakdown pulses. The conditions that favour hemi-fusion as opposed to complete fusion have been investigated. The observations made indicate that the complete electrofusion of erythrocytes, which are in close contact before exposure to breakdown pulses, is mediated by an intermediate structure that is stable in certain circumstances. This intermediate may be a shared bilayer that is comparable to the transient structures which have been observed ultrastructurally in some naturally-occurring fusion reactions of biological membranes, and to the more stable bilayer intermediates which are formed when planar phospholipid bilayers fuse in model systems (Neher, 1974; Melikyan et al., 1983; Horn, 1984; Fisher & Parker, 1984).

2.2 Materials and Methods

6-Carboxyfluorescein diacetate, and the carbocyanine, Dil-C<sub>16</sub>(3) (1,1-dihexadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate) were from Molecular
Probes, Pronase was from Serva, erythritol was from Sigma, and histidine was from BDH.

Human blood was withdrawn into a solution of ACD (Acid Citric Dextrose: 113.8 mM Glucose, 29.9 mM Trisodium citrate, 72.6 mM Sodium chloride, 2.8 mM Citric acid, pH 6.4) in the ratio of 5:1 (v/v) and mixed well, then the blood was washed at least three times with a buffered salt solution (124 mM NaCl, 40 mM Hepes, pH 7.4), centrifuged at a 1700 rpm (400xg) for 8 minutes and leucocytes were removed. The packed human erythrocytes in the buffered salt solution were stored at 4°C. 5 μl of a solution of carboxyfluorescein diacetate (20 mg/ml in acetone) (Goodall & Johnson, 1982) and 15 μl of a solution of Dil-C16(3) (2.5 mg/ml in ethanol) were added to the buffered salt solution (0.9 ml) and mixed. Packed, washed erythrocytes (0.1 ml) were added to this suspension, incubated for 20 min at 37°C, washed at least three times with the buffered salt solution. Then the labelled packed erythrocytes were mixed with unlabelled packed cells in the ratio of 1:9 (v/v) and stored at 4°C.

For electrical breakdown, 5 μl of the packed cells (labelled : unlabelled = 1 : 9) were mixed with 950 μl of a solution containing (150 or 250 mM) erythritol, 1 mg/ml Pronase (Zimmermann et al., 1981) and 10 mM histidine (conductivity around 286 μS/cm). The presence of Pronase stabilises cells against the a.c. field and d.c. field pulses: this appears to result from proteolytic action on the cells and the presence of contaminating Ca²⁺ ions in commercial preparations of Pronase (Ohno-Shosaku & Okada, 1985). 40 μl of the erythrocyte suspension in erythritol solution was placed in a chamber in which the electrodes were 260-280 μm apart, unless otherwise stated. At 1.5 min after the packed cells were added to the erythritol solution, the cells were aligned by dielectrophoresis in an a.c. field of 0.38 kV cm⁻¹ at 1.5 MHz from a
function generator (type TE7702, Toellner Electronic, Frankfurt). After a further 0.5 min, three square wave, electric field pulses (2.3-3.1 kV cm\(^{-1}\), 10-30 µs, as described in the text) were applied to the cells at 1 second intervals from a pulse generator (type 214B, Hewlett-Packard). The a.c. field was removed 1 min later. For cells exposed to the breakdown pulses at 25°C, cell counts were commenced after a further 2 min by which time diffusion of the Dil probe had reached equilibrium. For cells treated at 20°C, cell counts were commenced 5 min after exposure to the breakdown pulses.

Fluorescence microscopy for estimating the incidence of hemi-fusion was undertaken with Zeiss Standard microscope, fitted for epi-fluorescence, with a Neofluar 40/0.75 ph2 objective and an HBO 50 W mercury lamp. Two filter cassettes (BP 450-490/BP 520-560, and BP 546/12/LP 590) were used to view the carboxyfluorescein and Dil-C\(_{16}\)(3) probes, respectively. The electrode chamber was mounted on a heated stage, connected to a circulating water bath, the temperature of solutions used was controlled by a heating block, and room temperature was controlled by air-conditioning.

Visual fields were selected randomly for cell counting. Hemi-fused cells were inspected under both phase contrast and fluorescence microscope, and lysed cells were excluded from the counts. Single cells were also excluded. Between 100 and 200 cells (usually > 150) were counted under each condition. The incidence of hemi-fusion, \(\pm\) S.D\%., for 4-13 separate experiments (see Table I), was calculated by the formula \(\{(A-B)/A\} \times 100\%\), where \(A\) was the number of cells containing the Dil-C\(_{16}\)(3) probe, and \(B\) was the number of cells containing the carboxyfluorescein probe.

For freeze-fracture electron microscopy, a stainless steel pipetting annular electrode chamber (Kruss GmbH, Hamburg) with an electrode gap of 500 µm was used with a Zimmermann Cell Fusion System (GCA Corp., Chicago) at room temperature.
5 µl of the unlabelled, packed cells was added to 950 µl of the solution of 250 mM erythritol, 10 mM histidine, 1 mg ml⁻¹ pronase. 490 µl of the cells suspension was pipetted into the annular electrode chamber. At 1 min after the packed cells were added to the solution of erythritol, the cells were exposed to an a.c. field of 0.16 kV cm⁻¹ at 1.5 MHz. After a further 0.5 min, a single square wave pulse (5 kV cm⁻¹, 60 µs) was applied. The a.c. field was removed 0.5 min later, and the cells were fixed after a further 5 min by incubation with an equal volume of 4% (v/v) glutaraldehyde in the erythritol pulsing medium at room temperature for 2 hours. The fixed cells were gently centrifuged (600 x g for 5 min), and resuspended in a solution of glycerol (30%) in water. Samples were subsequently frozen in Freon 22 and fractured in a Balzers BAF 301 freeze etch unit. The replicas were examined with a Phillips 201 electron microscope.

2.3 Results

2.3.1 Membrane fusion without cytoplasmic fusion (hemi-fusion)

When the voltage and duration time of the breakdown pulses are higher and longer than a threshold value, the exposure of erythrocytes to an electrical breakdown voltage is followed by the diffusion of fluorescent cytoplasmic probe 6-carboxyfluorescein from labelled to unlabelled cells. The fluorescent membrane probe Dil, also diffuses into unlabelled cells. These phenomena are consistent to the observations made by other investigators (Sowers, 1984; 1985; Ahkong & Lucy, 1986) and showed the induction of cell fusion by electrical breakdown pulses.

Human erythrocytes can be reversibly aligned in pearl chains, perpendicular to the electrodes, by dielectrophoresis in an a.c. field. The cells are not fused by the a.c.
field provided the field strength is below 0.7 kV cm$^{-1}$ (Stenger & Hui, 1986). Exposure of erythrocytes in such chains to high voltage d.c. breakdown pulses induces some of the cells to fuse. When the a.c. field is subsequently removed, the pearl chains are normally dispersed by thermal currents in the fusion chamber, yielding randomly distributed fused and unfused cells. Under certain conditions, however, cells in the pearl chains are irreversibly attached to one another by the breakdown pulses, even though not all of them are fused. Such chains, which contain both fused and unfused cells, are not dispersed when the a.c. field is removed. Instead, they drifted randomly in the chamber, as shown by Ahkong and Lucy (1988) and also by phase contrast microscopy (Fig. 2.3 A).

In the cell chains that were irreversibly attached to their neighbours by breakdown pulses, without being fused, the two fluorescent probes behaved in different ways. It was observed that the membrane probe, Dil, diffused from labelled to unlabelled cells without the cytoplasmic probe diffusing into unlabelled cells or leaking into the medium. An example of this behaviour is illustrated by the fluorescence micrographs shown in Figs.2.1B-2.1D. These micrographs were taken, in the absence of the a.c. field, 10-15 min after the erythrocytes had been exposed to breakdown pulses. In Fig. 2.1B, the cytoplasmic label is restricted to two adjacent cells in a stabilized chain, but the membrane probe in Fig. 2.1C has diffused into at least one cell to the left and into two cells to the right of the cytoplasmically-labelled pair. The erythrocytes into which the membrane probe has diffused, but which do not contain the cytoplasmic probe, are clearly visible by phase contrast microscopy in Fig.2.1D and they were thus not lysed by the breakdown pulse.
Figure 2.3 Fluorescence and Phase-contrast micrographs of human erythrocytes which were photographed, in the absence of the AC field, about 15 min after the application of three square wave, 28-µs DC pulses (at 3.2 kV/cm) at 1-s intervals (in a chamber in which the electrodes were 280 apart). The cells were initially aligned in pearl chains, perpendicular to the electrodes, by dielectrophoresis in a solution of erythritol (250 mM) containing 1 mg/ml pronase and 10 mM histidine, at 25°C. Panel A shows that, after exposure to a DC breakdown pulse and subsequent removal of the AC field, the cell remained attached to one another in randomly orientated chains (arrows). Panel B shows cells labelled with the cytoplasmic fluorophore, carboxyfluorescein; this fluorophore is restricted to two cells. Panel C shows cells labelled with the membrane fluorophore, Dil. The arrowed cell, which is immediately to the right of the carboxyfluorescein-labelled pair, (and other cells that similarly do not contain carboxyfluorescein) are labelled with Dil. Panel D (a phase contrast micrograph) shows that carboxyfluorescein has not been lost from the arrowed cell as a consequence of lysis. Magnification: ×440.
The behaviour of fluorescent probes in the stabilised pearl chains of unfused cells thus indicates that membrane fusion (hemi-fusion) occurred without cytoplasmic fusion in response to the breakdown pulses.

2.3.2 Effects of changes in the electrical parameters on hemi-fusion

When erythrocytes at 25°C in 250 mM erythritol solution were subjected to applied breakdown pulses of 2.7 kV cm⁻¹, 20 μs, the cells in the pearl chains formed by the a.c. field were irreversibly attached to one another by breakdown pulses and were not dispersed by thermal and mechanical motion after the a.c. field was removed. It was observed that an increase in the duration time of the electric field pulses from 20 μs to 30 μs resulted in a small decrease in hemi-fusion (Table 2.1) and a small increase in complete fusion. When the duration time of the pulses was conversely shortened to 10 μs, there was little diffusion of either of the two probes, the pearl chains of cells were completely dispersed by thermal and mechanical motion after the a.c. field was removed, and both hemi-fusion and complete fusion were negligible. Pulses of approximately 20 μs were therefore optimal for inducing hemi-fusion.

With 20 μs pulses an increase in the breakdown voltage from 2.7 to 3.1 kV cm⁻¹ slightly decreased the incidence of hemi-fusion (Table 2.1), and increased complete fusion. When the breakdown voltage was decreased to 2.3 kV cm⁻¹ with 20 μs pulses, there was little diffusion of either of the two probes, the pearl chains of cells were readily dispersed in the absence of the a.c. field and both hemi-fusion and complete fusion were again negligible. It thus appears that when the duration time and the voltage of the applied pulses are above threshold values, an increase in the duration time of the pulses, or an increase in the voltage of the applied pulses, results in an increase in the number of completely fused cells and a decrease in hemi-fusion. Conversely, if the
value of either of these parameters is decreased below threshold values, the applied
electrical energy is insufficient even to induce hemi-fusion.

2.3.3 Effects of osmolarity and cell swelling on hemi-fusion

Potassium ions leak from human erythrocytes within a few seconds of their
exposure to an electrical breakdown pulse (Kinosita & Tsong, 1977a). This causes cell
shrinkage which is followed by cell swelling of the permeabilised cells. In the above
experiments it was observed that cells that were fused by breakdown pulses, as judged
by the transfer of carboxyfluorescein from labelled to unlabelled cells, were usually
extensively swollen after electrical breakdown. By contrast, hemi-fused cells were
usually much less swollen, or even shrunken.

Table 2.1

<table>
<thead>
<tr>
<th>Applied voltage (kV cm⁻¹)</th>
<th>Duration pulses of (μs)</th>
<th>Osmolarity (mM erythritol)</th>
<th>Temp. (°C)</th>
<th>Incidence of hemi-fusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.7</td>
<td>10</td>
<td>250</td>
<td>25</td>
<td>negligible</td>
</tr>
<tr>
<td>2.7</td>
<td>20</td>
<td>250</td>
<td>25</td>
<td>50.9 ± 3.8 (13)</td>
</tr>
<tr>
<td>2.7</td>
<td>30</td>
<td>250</td>
<td>25</td>
<td>42.0 ± 4.1 (7)</td>
</tr>
<tr>
<td>2.3</td>
<td>20</td>
<td>250</td>
<td>25</td>
<td>negligible</td>
</tr>
<tr>
<td>3.1</td>
<td>20</td>
<td>250</td>
<td>25</td>
<td>40.6 ± 3.3 (11)</td>
</tr>
<tr>
<td>2.7</td>
<td>20</td>
<td>150</td>
<td>25</td>
<td>23.4 ± 2.8 (8)</td>
</tr>
<tr>
<td>2.7</td>
<td>20</td>
<td>250</td>
<td>20</td>
<td>10.6 ± 1.3 (4)</td>
</tr>
<tr>
<td>2.7</td>
<td>20</td>
<td>250</td>
<td>30</td>
<td>59.2 ± 1.2 (5)</td>
</tr>
<tr>
<td>2.7</td>
<td>20</td>
<td>250</td>
<td>37</td>
<td>approx. 60</td>
</tr>
</tbody>
</table>

The effect of a decrease in the osmolarity of the erythritol solution, from 250
mM to 150 mM, on the incidence of hemi-fusion was determined at 25°C, under
optimal electrical conditions for hemi-fusion (breakdown pulses of 20 μs, and a voltage of 2.7 kV cm⁻¹). In 150 mM erythritol solution, the cytoplasmic probe carboxyfluorescein diffused from labelled cells to many unlabelled cells along the pearl chains formed by the a.c. field. The membrane probe Dil-C₁₆(3) of labelled cells diffused to unlabelled cells to a similar extent or to 1-2 unlabelled cells further along the pearl chain of cells and this indicated a high fusion yield. In the 250 mM erythritol solution, the cytoplasmic probe carboxyfluorescein diffused to fewer unlabelled cells along the pearl chains formed by the a.c. field, which indicated a lower fusion yield, whereas the membrane probe Dil-C₁₆(3) of labelled cells diffused to more unlabelled cells than the carboxyfluorescein probe. So the incidence of hemi-fusion with cells in 250 mM erythritol was found to be approximately double that with the more swollen cells in 150 mM erythritol (Table 2.1). This observation further indicates that there is an inverse relationship between the conditions that favour hemi-fusion and that permit complete fusion if the electrical parameters are above threshold values.

2.3.4 Effect of temperature on hemi-fusion

The incidence of hemi-fusion was influenced strongly by changes in temperature. Under optimal electrical and osmotic conditions (breakdown pulses of 20 μs, 2.7 kV cm⁻¹, 250 mM erythritol solution), the incidence of hemi-fusion at 25°C (50.9 ± 3.8%) was approx. 5-fold greater than that at 20°C (10.6 ± 1.3%) (Table 2.1). The diffusion of the membrane probe Dil-C₁₆(3) from labelled to unlabelled human erythrocytes, which are fused by electric field pulses, has been investigated previously by Sowers (1985). He recorded the movement of the fluorescent label by microscopy, and obtained average lateral diffusion coefficients of 3.8x10⁻⁹ and 8.1x10⁻⁹ cm²s⁻¹ in isotonic phosphate buffer at pH 7.4, at 23-25°C and 35-37°C, respectively. In our
experiments, it was found that the diffusion of Dil-C16(3) from labelled to unlabelled cells was slower at 20°C than at 25°C. After the application of breakdown pulses, the cytoplasmic probe carboxyfluorescein diffused almost-instantaneously from labelled cells to many unlabelled cells along the pearl chains formed by an a.c. field at 20°C even in 250 mM erythritol solution, whereas the diffusion of Dil-C16(3) probe was much slower. To avoid the artifact from slower diffusion of Dil-C16(3) probe, cell counts were commenced at 20°C only when diffusion of the probe had reached equilibrium.

It was also noteworthy that a high proportion of the cells (both fused and unfused) were swollen 10 min after the exposure to breakdown pulses at 20°C (Fig.2.4A), whereas the cells (many of which were hemi-fused) were extensively shrunken 10 min after exposure to breakdown pulses at 25°C (Fig.2.4B). With time, the shrunken cells swelled slowly, and they exhibited extensive swelling after 1 hour at 25°C.

A further, but relatively small increase in hemi-fusion, was observed at 30°C (Table 2.1). Due to considerable bleaching of the Dil probe at 37°C and the leaking of carboxyfluorescein from labelled cells into the background, it was difficult to estimate the incidence of hemi-fusion accurately at this temperature. However, hemi-fusion appeared to be no greater at 37°C than 30°C.

It has been reported that the membrane skeleton of human erythrocytes inhibits the electrofusion of human erythrocytes, and that the incidence of fusion increases sharply when the proteins of the skeleton are denatured by heating the cells to 50°C (Glaser & Donath, 1987). The effect of heating erythrocytes at about 50°C, which were hemi-fused at 25°C, was therefore studied. Carboxyfluorescein was observed to diffuse from labelled, hemi-fused cells into adjacent unlabelled cells, showing that hemi-fused
Figure 2.4 Bright-field light micrographs of human erythrocytes, in the absence of the AC field, 10 min after the application of three square wave, 28-μs DC pulses (at 3.2 kV cm⁻¹) at 1-s intervals (with the electrodes 280 μm apart). The cells were initially aligned by dielectrophoresis in the medium used in Fig.1 Cells in panel A were exposed the breakdown pulses at 20°C, and a high proportion of them (both fused and unfused) are swollen. Cells in panel B (most of which are hemi-fused) were exposed to the breakdown pulses at 25°C, and they are extensively shrunken. Magnification: ×200.
cells proceed to complete, cytoplasmic fusion at this temperature. Probe bleaching and movements of the erythrocytes at 50°C also prevented micrographs of the phenomenon from being obtained.

2.3.5 *Ultrastructural changes associated with hemi-fusion*

To investigate the ultrastructure of the hemi-fused cells, human erythrocytes in 250 mM erythritol solution were aligned by dielectrophoresis in an annular electrode chamber, exposed to a single breakdown pulse (5 kV cm⁻¹, 60 μs), and then prepared for freeze-fracture electron microscopy as described in Materials and Methods. under these conditions, very few cells were completely fused by the breakdown pulses.

During these experiments it was observed that the linkages between hemi-fused cells are easily disrupted, and it was therefore necessary to centrifuge chains of hemi-fused cells gently in order to preserve them for electron microscopy. Figs. 2.5 and 2.6 are electron micrographs which show the appearance of freeze-fractured cells that are closely apposed and may be hemi-fused. In Fig. 2.5, patchwork-like structures (curled arrows) are present where the fracture plane has jumped from the E-face down to the P-face, and back again. Irregular protrusions occur on the P-face floor of the patches. More defined, small protrusions (arrow heads), approx. 18 nm in diameter, are visible on the P-face of the lower cells in both Figs. 2.5 and 2.6. The dimensions of these protrusions appear to match depressions (arrows) that are present on the apposed E-face of the upper cell in Fig. 2.6. These protrusions and depressions may represent sites of attachment and membrane continuity in hemi-fused cells.
**Figure 2.5** An electron micrograph of freeze-fractured human erythrocytes that are closely apposed and may be hemi-fused, which was prepared as described in materials and Methods. Patchwork-like structures (curled arrows) are present where the fracture plane has jumped from the E-face down to the P-face, and back again. Irregular protrusions occur on the P-face floor of the patches. More defined, small protrusions (arrow heads), approx. 18 nm in diameter, are also visible on the P-face of the lower cell. Magnification: ×53000.

**Figure 2.6** An electron micrograph of another pair of freeze-fractured human erythrocytes that may be hemi-fused. The protrusions (arrow heads), approx. 18 nm in diameter, that are present on the P-face of the lower cell appear to match depressions (arrows) seen on the apposed E-face of upper cell. These features may represent the sites of attachment and membrane continuity in hemi-fused cells. Magnification: ×82000.
2.4 Discussion

It was found that hemi-fusion only occurred when the cells in the pearl chains became irreversibly attached to one another by the breakdown pulses. Human erythrocytes in the same solutions aligned by an a.c. field but without being exposed to breakdown pulses were observed for long time after the removal of the a.c. field, the pearl chains of cells were dispersed by thermal movement and no aggregation was found, the possibility that the irreversibly attachment of cells in the pearl chains is resulted from the effects of low ionic strength mentioned by Scheurich et al. (1980) and Zimmermann (1982) thus was excluded. Neither DiI nor the carboxyfluorescent probe diffused from labelled to unlabelled cells in the pearl chains formed by an a.c. field alone, so the possibility that the diffusion of the DiI probe in hemi-fused cells results from the direct exchange of the membrane probe between the two closely apposed erythrocytes was excluded. The protrusions and depressions in Freeze-fracture micrographs (Fig. 2.5 and Fig. 2.6) may represent sites of attachment and membrane continuity in hemi-fused cells. Above evidence suggests that the more extensive diffusion of the DiI probe than of the carboxyfluorescein probe in cells in the pearl chains irreversibly attached by breakdown pulses under certain conditions represents the fusion of the outer monolayers of apposed human erythrocyte membranes without cytoplasmic fusion (hemi-fusion).

Sowers (1984) also used the membrane probe DiI-C_{16}(3) and the cytoplasmic probe FITC-dextran to label human erythrocytes ghost membranes separately (not double-labelling) and reported that human erythrocyte ghosts, orientated in pearl chains by an a.c. field, were occasionally attached to one another by breakdown pulses since subsequent removal of the a.c. field gave rise to randomly orientated, but indefinitely
stable, pearl chains. However, in his experiments, neither the cytoplasmic fluorophore (FITC-dextran) nor the membrane probe (DiI) diffused from labelled to unlabelled ghosts in the stabilised pearl chains. It was therefore suggested that the glycocalyx and cytoskeleton were involved in the attachment of the ghost plasma membranes to one another. By contrast, both the octadecyl rhodamine probe used in preliminary experiments on electrically-induced, hemi-fusion (Ahkong & Lucy, 1988), and the DiI probe employed in the present work, diffused from labelled to unlabelled cells in pearl chains that were stabilised by breakdown pulses. Sowers (1988) also found an apparent discrepancy between the contents-mixing assay and the membrane-mixing assay in a separate experiment. In his experiments, in contrast to our present results, the contents-mixing events between adjacent single cells usually exceeded membrane-mixing events by a considerable margin, and Brownian motion caused some of the groups of ghosts in which contents mixing occurred to eventually separate from one another. He believed that the discrepancy was due to the contents-mixing events not representing fusion.

Although it is possible that the cytoplasmic probe may have diffused directly from labelled cells to nearby unlabelled cells without any attachments being established, this artifact can be easily avoided by not counting content mixing between adjacent single cells (i.e. counting only cells in pearl chains).

It has been reported by Chang (1989) that both membrane fusion and cytoplasmic fusion are observed when electric field pulses of radio-frequency are applied to human erythrocytes that are aligned in chains by dielectrophoresis. In this work DiI-C\textsubscript{16}(3) was used to monitor membrane continuity, and membrane fusion was regarded as an abortive fusion process in which the fusion partners are able to fuse their membrane lipid bilayers but fail to establish a cytoplasmic bridge. Cell fusion was,
however, determined by visual observation of the cytoplasmic merging of adjacent cells through the optical microscope, and experiments with FITC-dextran have shown that it is possible for cytoplasmic continuity to be induced between adjacent cells by a d.c. breakdown pulse in the absence of visible cytoplasmic merging (Sowers, 1984).

The electrical breakdown of cell membranes has been interpreted in terms of an electro-mechanical model which postulates that membrane thickness depends on the voltage across the membrane and the applied pressure (Zimmermann et al., 1977; Zimmermann et al., 1980). Zimmermann (1982) has also suggested that pores in plasma membranes, which are produced by electrical breakdown, mediate cell fusion when they are induced in closely apposed membranes. However, from ultrastructural observations on the electrically-induced fusion of human erythrocytes aligned by dielectrophoresis, Stenger and Hui (1986) have concluded that fusion sites arise where electrically damaged regions of membrane (point defects) make contact. Lucy and Ahkong (1988) also suggested that a breakdown pulse, which is just below the threshold that is necessary to induce sufficient membrane thinning for complete fusion, may nevertheless have enough energy to compress the adjacent plasma membranes of aligned cells into shared bilayers at the points of very close contact, i.e. induce hemi-fusion. It is possible that the rupture of the shared bilayers at the point of contact induced by breakdown pulses, may be assisted by osmotic forces, occur so quickly that cell fusion is usually virtually instantaneous with electrical breakdown (Lucy, 1986).

Schmitt and Zimmermann (1989) have concluded, however, that neither pressure, nor osmotic gradients, nor the increase in volume of cells in hypo-osmolar solutions, contribute directly to the mechanism of electrofusion since they found that treatment of myeloma cells with hypo-osmotic solutions enhanced the electrofusion field
even when the cells were returned to iso-osmolar conditions before being subjected to electrofusion. They suggested that the increase in fusion yield is due, instead, to the small increase in membrane permeability associated with the swelling process and/or to the dissolution of membrane skeleton proteins caused by osmotic stress.

Pores that are induced in cell membranes by an electrical breakdown pulse reseal increasingly rapidly between 10°C and 37°C. It has been reported that the electrically-induced leak permeability of human erythrocytes decreases by 85% within 15 min at 25°C and, at breakdown voltages of approx. 3 KV cm\(^{-1}\), the number of erythritol-permeable pores may average only one per cell (Deuticke & Schwister, 1989, book; Schwister & Deuticke, 1985). The marked increase in electrical hemi-fusion (and decrease in complete fusion) at 25°C, by comparison with that seen at 20°C in our experiments, may therefore have been due to rapid (although incomplete) resealing of permeabilised cells at the higher temperature. In a long pearl chain of cells formed by a.c. field, after the electrical breakdown, the carboxyfluorescein probe in a labelled cell diffused through the pores induced by the breakdown pulses. At 20°C, the pores resealed slower, so carboxyfluorescein probe can diffuse to many cells along the pearl chain through the unsealed pores. At 25°C, the pores resealed quicker, before carboxyfluorescein probe reached many unlabelled cells in the pearl chains, the size of the pores of the pulsed cells has been too small to permit the probe to pass through. So carboxyfluorescein probe diffused to less unlabelled cells at 25°C than at 20°C, this may explain why the hemi-fusion yield were higher at 25°C than at 20°C. This interpretation is supported by our observation that, 10 min after exposure to the breakdown pulses, erythrocytes at 20°C were mostly swollen whereas those at 25°C were mostly shrunken.
Since hemi-fused erythrocytes occasionally fused completely on heating to 50°C, hemi-fused cells can give rise to completely fused cells. A delayed cell fusion has also been observed (monitored by the diffusion of carboxyfluorescein) when electrically-permeabilised erythrocytes are subsequently allowed to swell in 200 mM erythritol (Lucy, 1986). It is possible that the electrofusion of cells is mediated via a transient, hemi-fused state.

The depressions on the E-face and protrusions on the P-face of the membranes in the present work are closely similar to the transient point defects (diameter 20-50 nm) that were observed by Stenger and Hui (1986) between 2 and 10 s after exposing aligned human erythrocytes to a breakdown pulse. These investigators suggested that the defects were related to a transient form of membrane contact or adhesion that led, within 10 s, to the formation of permanent lumina between fusing cells. Virtually no point defects were observed 10 s after the breakdown pulse. The corresponding structures observed in our experiments were, however, still present 5 min after the breakdown pulse. They may mediate hemi-fusion by breakdown pulses that are insufficient to induce complete fusion, and may also mediate complete cell fusion (whether it occurs 10 s after the breakdown pulse or, under certain conditions, many minutes later).

It is relevant that patchwork-like structures where the fracture plane jumped reversibly between two closely apposed membranes, as in the present work, were observed in freeze-fractured preparations of mitochondria that were quenched from 37°C (Van Venetie & Verkleij, 1982). Bulges and pits (diameter 25 nm) were also seen that may have represented attachment sites of the inner and outer membrane. Contact points between the two mitochondria membranes were interpreted in terms of a semi-
fusion model, but it was not possible to discriminate between semi-fusion with, or without an inverted micelle (or Hn-tube), at the points of contact.

Ultrastructural studies on the fusion reactions of biomembrane (Palade & Bruns, 1968; Palade, 1975; Pinto da Silva & Nogueira, 1977; Kalderon & Gilua, 1979; Kanchanapoom & Boss, 1986) have indicated that an intermediate single bilayer (hemi-fusion) may occur, but such intermediates are very transitory structures. In the present investigation it has been shown that the electrical breakdown of human erythrocytes under specific experimental conditions favours stable, hemi-fused cells at the expense of complete cell fusion, and that heating the hemi-fused cells occasionally results in complete fusion. This behaviour resembles that of human erythrocytes when they exhibit membrane fusion without cytoplasmic fusion on dehydration with 40% poly(ethylene glycol), followed by complete fusion on subsequent swelling in isotonic buffer (Ahkong et al., 1987). In this case, complete cell fusion is preceded by the formation of stable, hemi-fused cells. The intermediate structure present in these cells may be some form of shared bilayer, which is structurally similar to the transient bilayer that have been observed in naturally-occurring fusion reactions of biological membranes. However, unlike these intermediates, there are no immediate stress that rapidly complete the fusion reaction. In this respect, the intermediate in the hemi-fused erythrocytes resembles the more stable bilayer structures which mediate the fusion of planar phospholipid bilayers in model systems (Neher, 1974; Melikyan et al., 1983; Horn, 1984; Fisher & Parker, 1984; Helm et al., 1989), but the possibility that a transitory Hn hexagonal phase or other non-bilayer configuration may occur in the hemi-fused erythrocytes cannot be excluded.
Summary

Evidence was found from both fluorescence microscopy and freeze-fracture electron microscopy for hemi-fusion in the electrofusion of human erythrocytes. A membrane probe (DiI-C₁₀(3)) and a cytoplasmic probe (6-carboxyfluorescein) were used to investigate the behaviour of doubly-labelled human erythrocytes which were aligned in chains by dielectrophoresis and then exposed to high voltage breakdown pulses. Some of the cells were fused by the pulses, as shown by diffusion of both membrane and cytoplasmic probes from labelled to unlabelled cells. With other cells, the membrane probe diffused into unlabelled cells after the breakdown pulses, without the cytoplasmic probe diffusing into unlabelled cells or leaking into the medium. Membrane fusion (hemi-fusion) thus occurred without cytoplasmic fusion in these erythrocytes. Such cells were irreversibly, but fragilely, attached to their neighbours by the breakdown pulses. There was an inverse relationship in such cells between conditions that permit complete fusion and those that favour hemi-fusion, with respect to breakdown pulse length, breakdown voltage and, in particular, osmolarity and temperature. The incidence of hemi-fusion in 250 mM erythritol was twice that in 150 mM erythritol, and hemi-fusion was 5-fold greater at 25°C than at 20°C. Hemi-fused erythrocytes occasionally fused completely on heating to 50°C, demonstrating that hemi-fusion can proceed to and might precede complete cell fusion. Freeze-fracture electron micrographs of preparations of hemi-fused cells revealed long-lived, complementary depressions and protrusions on the E- and P-fracture faces of tightly apposed cells respectively, which may mediate hemi-fusion. It is possible that the fusion of human erythrocytes by electrical breakdown pulses may involve an intermediate, shared bilayer structure.
Chapter Three
Phospholipid asymmetry, divalent cations, and osmotic swelling in electrically-induced lysis, cell fusion, and giant cell formation with human erythrocytes

3.1 Introduction

Exposure of acidic phospholipids on the outside of sickled erythrocytes (Middelkoop et al., 1988), and platelets (Verhallen et al., 1987, 1988) yields procoagulant surfaces that facilitate the conversion of prothrombin into thrombin. The catalytic potential of such surfaces provides a semi-quantitative measure of the exposed acidic phospholipids (Verhallen et al., 1987, 1988; Gerads et al., 1988). Baldwin et al. (1990) observed that an increase in the procoagulant activity of human erythrocytes is associated with fusion of these cells that is induced by the permeant molecule poly(ethylene glycol) 400 in the presence of Ca^{2+}. Erythrocytes that were incubated with ionophore A23187, subtilisin, and Ca^{2+} also developed procoagulant activity, equivalent to a complete loss of phospholipid asymmetry, and they fused on subsequent exposure to a hypotonic medium. From these experiments Baldwin et al. (1990) concluded that a translocation of phosphatidylserine (PS) to the outer leaflet of the plasma membrane plays an important role in fusion protocols that involve cell swelling.

Recent reports from other laboratories have also indicated that changes in the phospholipid asymmetry of plasma membranes are associated with cell fusion (Tullius et al., 1989; Huang & Hui, 1990). By contrast, although fusion of vesicular stomatitis virus with lipid-symmetric human erythrocyte ghosts is more rapid than fusion with lipid-asymmetric ghosts, the susceptibility to fusion appears to be related to the packing
characteristics of the target membrane rather than to any particular phospholipid (Herrmann et al., 1990).

Dressier et al. (1983) found that the asymmetric distribution of phosphatidylethanolamine (PE) in the plasma membrane of human erythrocytes was completely lost in ghosts prepared by colloid-osmotic lysis after electric breakdown and resealing, although PS was much less affected. No experiments on the fusion of cells exposed to the electric breakdown pulses were reported, but a possible mechanistic link between the enhancement of phospholipid flip-flop and electrically-induced cell fusion was discussed. It has been observed that electric field pulses induce the formation of a ‘long-lived fusogenic state’ (Sowers, 1986) or ‘transient permeant structures’ (Teissié & Rols, 1986) that enable cell fusion to occur when contact between cells is established after they have been subjected to field pulses. Changes in the phospholipid asymmetry of plasma membranes might contribute to such states or structures (Tsong, 1990). Experiments were therefore undertaken to investigate possible relationships between the surface exposure of acidic phospholipids and cell fusion in human erythrocytes that are exposed to electric field pulses. Although it has been reported that the prothrombinase assay detects PE almost as well as PS in mixtures with PS (Gerads et al., 1990), the assay was shown to be highly-specific for PS under the conditions of current experiments in this chapter by Jocelyn Baldwin (see Song et al., 1993). Consequently, it is concluded that the enhanced procoagulant activity of human erythrocytes after electrical breakdown observed in the present work is due to a movement of PS from the inner to outer leaflet of the plasma membrane.

In early work on the fusion of erythrocytes by the Sendai virus, Mn$^{2+}$ virtually prevented haemolysis induced by the virus and permitted a high fusion index to be
obtained (Zakai et al., 1974). It was therefore suggested that bivalent metal cations facilitate cell fusion by inhibiting cell lysis. More recently it was observed that, when monolayers of human erythrocytes were induced to swell by the entry of small molecules, lysis occurred without cell fusion in the absence of Ca$^{2+}$, whereas in the presence of Ca$^{2+}$ many of the swelling cells fused before they lysed (Ahkong & Lucy, 1988; Baldwin et al., 1990). It is reported in this chapter that Mn$^{2+}$, Ca$^{2+}$, and Ba$^{2+}$ similarly inhibit cell lysis and facilitate cell fusion with human erythrocytes that are subjected to electrical breakdown. In the light of the relationships found between the surface exposure of PS and cell fusion in erythrocytes subjected to electrical breakdown in the present work, it is suggested that interactions with PS that is exposed at the cell surface may enable Mn$^{2+}$, Ca$^{2+}$ and Ba$^{2+}$ to inhibit lysis and facilitate cell fusion, at least with erythrocytes that are subjected to electrical breakdown.

Although cell fusion is initiated within microseconds of electrical breakdown, the time taken to achieve the final spherical shape was found to vary in early work from seconds to 60 min, depending on the species of cell, as well as on the number of cells being fused. It was proposed that the time needed for complete fusion in differing cell types depends on their membrane fluidities, and on the properties of the cytoskeleton within the cells (Zimmermann, 1982; Zimmermann et al., 1981). In many instances, and particularly in the case of fused mammalian cells, the speed with which the spherical shape was assumed appeared to depend on the presence of ions, with 1 mM Ca$^{2+}$ being especially effective. By contrast, no evidence was found for the involvement of osmotic process in the rounding-up of fused cells (Zimmermann, 1982). Relatively little work has since been done on the factors that affect the morphology of intact cells after electrically-induced cell fusion, although it has recently been concluded that the
spectrin network and a non-osmotic force controls the morphology of erythrocyte ghosts fused by electrical breakdown (Chernomordik & Sowers, 1991). The present observations indicate that osmotic swelling, which results from the entry into cells of sugar molecules via electropores in the plasma membranes, is actually responsible for the rounding-up of human erythrocytes into giant cells, following exposure to electrical breakdown pulses in low ionic strength media. It is suggested that, as with erythrocytes treated with Sendai virions, osmotic swelling appears to be the driving force that results in permeabilised cells, in which membrane fusion sites are present, being able to expand into giant cells.

It was also found that, in the presence of Zn\(^{2+}\) ions, erythrocytes do not shrink on electrical breakdown in low ionic strength media, and that they subsequently swell very rapidly into giant cells. This may result from an interaction of Zn\(^{2+}\) ions with cysteine groups in membrane proteins that prevents the loss of ions that would otherwise occur on electrical breakdown.

### 3.2 Materials and Methods

#### 3.2.1 Materials

Bovine blood coagulation factors (factor Xa, prothrombin, thrombin) were from Sigma Chemical Co. Bovine factor Va was from Diagnostic Reagents Ltd. The chromogenic substrate for thrombin, S2238, (H-D-phenylalanyl-L-pipeccolyl-L-arginine-p-nitroanilide dihydrochloride), was from KabiVitrum (Stockholm, Sweden).

#### 3.2.2 Methods

Unless otherwise stated, assays for prothrombinase were performed in duplicate, and each experiment was undertaken at least three times. In each case, the results
shown are from one representative experiment.

3.2.2.1 Exposure of erythrocytes to electrical breakdown pulses

Human erythrocytes were washed and freed from leucocytes as described in Chapter 3. 5 μl of a packed suspension of the cells was added to 950 μl of a solution of sucrose or other sugar (250 mM unless otherwise stated), containing 1.1 mM of a divalent cation (Ca\(^{2+}\) unless otherwise stated) and 10 mM histidine, which had a conductivity of 235 \(\mu\)S·cm\(^{-1}\). Ca\(^{2+}\) was present in the medium to stabilise the erythrocytes against the alternating and the DC field pulses (Ohno-Shosaku & Okada, 1985). In the absence of Ca\(^{2+}\), the cells lysed when the DC pulses were applied. A stainless steel pipetting annular electrode chamber (Kruss GmbH, Hamburg) with an gap of 500 μm was used with a Zimmermann Cell Fusion System (GCA Corp., Chicago). 1.5 min after adding the packed cells to the buffered sucrose, the erythrocytes were aligned by dielectrophoresis in an AC field of 0.16 kV·cm\(^{-1}\) at 1.5 MHz. after a further 0.5 min, three square wave, electric field pulses (of 99 μs, unless otherwise described in the text) were applied to the cells at 1 s intervals, and the a.c. field was removed 0.5 min later. The pulsed cell sample was removed from the electrode chamber, and portions were used to determine the cell fusion index, to investigate the number of cells in fused chains of erythrocytes, to determine cell lysis, and to investigate cell swelling, cell rounding, and the formation of giant cells. For the assay of procoagulant activity of the pulsed erythrocytes, the sample was divided into two portions. One portion was used to investigate cell fusion qualitatively; the other portion was assayed for prothrombinase activity and cell lysis. To avoid possible interference by carboxyfluorescein in the prothrombinase assay, quantitative assays of cell fusion were made separately. All experiments were done at 25°C unless otherwise
stated.

3.2.2.2 Assay of procoagulant activity and cell lysis

Samples (20 μl) of pulsed or unpulsed erythrocytes, or lysed cells, were added to 480 μl of the above buffered sucrose solution. 100 μl samples were then removed for the determination of procoagulant activity. 50 μl of a cocktail (containing factor Xa, factor Va, and CaCl₂) was added to each sample, and the mixture was incubated at 37°C for 2 min before adding 50 μl of a pre-warmed solution containing prothrombin and CaCl₂. The final concentrations in the assay were: 2 μM prothrombin, 4mM CaCl₂, 0.2 units per ml factor Xa, and 6 nM factor Va. Unless otherwise stated in the text, prothrombin was added to the cells 6 min after the electrical breakdown pulses. After various subsequent time intervals, 20 μl samples were removed, and thrombin formation was stopped by diluting them into 0.5 ml 50 mM Tris-HCl, 120 mM NaCl, 2 mM EDTA (pH 7.5) at room temperature. Chromogenic substrate S2238 was added to a final concentration of 150 μM, and the quantity of thrombin present was determined from the rate of change of absorbance at 405 nm using a calibration curve.

The remains of each sample of pulsed or unpulsed cell sample, was maintained at 37°C for 11 min (until the prothrombinase assays were completed), and then centrifuged at 2000 × g for 3 min. Cell lysis was estimated from the absorbance at 405 nm of the haemoglobin in the supernatant by comparing the absorbance values obtained with those from a preparation of cells which had been totally lysed, by adding 5 μl of packed cells to 950 μl of distilled water, and subsequently sonicated. The preparations of lysed cells that were obtained in this way gave values in the assay for the maximum procoagulant activity which were reproducible throughout the course of each experiment.
3.2.2.3 Cell fusion

Erythrocytes were labelled with 6-carboxyfluorescein as previously described (Ahkong et al., 1987), but the labelled cells were then mixed with unlabelled cells in the proportion of 1:4.5. Exposure of human erythrocytes to an electrical breakdown voltage is followed by the almost-instantaneous diffusion of 6-carboxyfluorescein from labelled to unlabelled cells (Ahkong & Lucy, 1986). The percentage of cell fusion was determined using a Nikon Diaphot-TMD microscope, fitted with a × 100 oil immersion objective, an HBO 100 W mercury lamp, and a B filter cassette to view the carboxyfluorescein probe by epifluorescence. Cells were counted on a microscope slide in randomly selected fields after their exposure to breakdown pulses. Between 100 and 450 cells (depending on the incidence of fusion) were counted in triplicate for each experimental condition. The percentage cell fusion was defined as \( B/A(\times 100) \), where \( B \) was the number of fused fluorescent cells in a pearl chain of erythrocytes, and \( A \) was the total number of cells counted. Fluorescent cells which were only adjacent to unlabelled cells in pearl chains were not included in \( B \). All cells in such chains were, however, included in \( A \). Single cells (labelled and unlabelled) were also included in \( A \). Pearl chains which had no fluorescent cells were not counted because the presence of cytoplasmic connections in such chains can not be established or excluded on a quantitative basis.

The mean number of cells in the pearl chains formed when (unlabelled) erythrocytes were subjected to electric breakdown pulses was calculated according to the following formula: \( \{(M^2 \times \alpha) + ([M-1]^2 \times \beta) + \ldots + 2^2 \times (\text{No. of 2-cell chains}) + (\text{No. of single cells})\}/\text{total number of cells} \), where \( M \) was the number of cells in the longest chain, \( \alpha \) was the number of cell chains containing \( M \) cells, and \( \beta \)
was the number of cell chains containing (M-1) cells. Between 200 and 400 cells were counted for each sample except that, because of the high percentage of cell lysis with erythrocytes that were subjected to electrical breakdown in the presence of Zn\(^{2+}\), only 110, 167 and 183 cells were counted in the triplicate experiments with this cation. Photographs of pearl chains of cells, and of the transformation of fused cells into giant cells, were obtained with a Nikon Diaphot-TMD microscope, fitted with a \(\times100\) oil immersion objective, using Technical Pan films (Kodak).

3.3 Results

3.3.1 Haemolysis, procoagulant activity, and fusion of erythrocytes exposed to breakdown pulses of increasing duration

Intact human erythrocytes in sucrose solutions had virtually no procoagulant activity (3.1% ± S.D. 2.1, \(n = 9\), of the procoagulant activity of lysed cells). When erythrocytes were incubated for 45 min at 37°C in sucrose solutions of decreasing osmolarity in previous work, their procoagulant activities increased in parallel with the cell lysis as a consequence of the increasing accessibility of PS in the inner leaflet of the plasma membrane (Baldwin et al., 1990). Erythrocytes are permeabilised by electrical breakdown pulses, and this results in colloid osmotic swelling that is followed by cell lysis (Kinosita & Tsong, 1977b). Consequently, erythrocytes that have been exposed to electrical breakdown pulses similarly exhibit procoagulant activity. In order to see if PS become accessible in pulsed cells as a primary consequence of electrical breakdown, rather than as a secondary consequence of cell lysis, it is desirable to minimise colloid osmotic swelling. A minimal release of haemoglobin (\(M, 65000\)) also indicates that factor Va (\(M, 330000\)), used in the prothrombinase assay, is unlikely to
have entered the pulsed cells. Minimal swelling and haemolysis were achieved by an appropriate choice of non-electrolyte in the medium used for pulsing the cells. Table 3.1 shows the percentage haemolysis observed, under the conditions of the experiments, 23 min after erythrocytes were subjected to three consecutive DC square wave pulses (5 kV·cm$^{-1}$) of 20 μs or 99 μs, in 250 mM solutions (conductivity 235 μS·cm$^{-1}$) of erythritol, mannitol, or sucrose (each containing 10 mM histidine and 1.1 mM Ca$^{2+}$). Although solutions of mannitol and erythritol are commonly used in the electrofusion of cells, it is clear that haemolysis of the treated erythrocytes was least in the sucrose solution. Erythrocytes were therefore pulsed in sucrose solution in all of the

<table>
<thead>
<tr>
<th>Solution</th>
<th>Duration of pulses (μs)</th>
<th>Haemolysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>250 mM erythritol</td>
<td>20</td>
<td>65.7 ± 4.5</td>
</tr>
<tr>
<td>250 mM mannitol</td>
<td>20</td>
<td>27.6 ± 4.0</td>
</tr>
<tr>
<td>250 mM sucrose</td>
<td>20</td>
<td>10.1 ± 1.1</td>
</tr>
<tr>
<td>250 mM erythritol</td>
<td>99</td>
<td>55.8 ± 2.5</td>
</tr>
<tr>
<td>250 mM mannitol</td>
<td>99</td>
<td>31.6 ± 4.3</td>
</tr>
<tr>
<td>250 mM sucrose</td>
<td>99</td>
<td>17.2 ± 2.1</td>
</tr>
</tbody>
</table>

Table 3.1 Colloid osmotic haemolysis of human erythrocytes after exposure to electrical breakdown pulses in differing non-ionic media.

experiments related to the assay of the procoagulant activity of the pulsed cells in the present work.
Figure 3.1 Human erythrocytes in a 250 mM sucrose solution, containing 1.1 mM Ca\textsuperscript{2+} and 10 mM histidine, were aligned in pearl chains by dielectrophoresis and subjected to three consecutive DC square wave pulses (5 kV cm\textsuperscript{-1}), of increasing duration time (0.5 to 99 \mu s), as described in Material and Methods. The percentage procoagulant activity (\textcircled{O}), percentage cell fusion (\texttriangle), and percentage haemolysis (\textbullet) were determined as described in Materials and Methods.

The effect of electrical breakdown pulses (5 kV cm\textsuperscript{-1}) of increasing duration, from 0.5 to 99 \mu s, on the procoagulant activity and the haemolysis of erythrocytes suspended in 250 mM sucrose solutions was investigated. Fig. 3.1 shows that the percentage procoagulant activity of the cells increased much more rapidly than the percentage lysis for pulse lengths between 0.5 \mu s and 20 \mu s. Further more, with 60 \mu s pulses about 58\% of the total prothrombinase activity was exposed although, judging from the percentage haemolysis, only about 15\% of the total prothrombinase activity.
was attributable to acidic phospholipids in the inner monolayer of lysed cells. Approximately 43% of the PS were therefore exposed at the surface of the treated cells. However, for reasons considered below in the Discussion section, this is probably an underestimate of the loss of phospholipid asymmetry in the pulsed cells.

With a breakdown voltage of 5 kV·cm⁻¹, pulses of only 2 μs duration were sufficient to induce a marked loss of phospholipid asymmetry in the treated erythrocytes (Fig. 3.1). Interestingly, much longer pulses were required for the induction of cell fusion (Fig. 3.1). It is thus apparent that in addition to the surface exposure of PS, other structural changes in the erythrocyte membrane, which are induced by relatively long electrical pulses, precede cell fusion.

3.3.2 Effects of the breakdown voltage on procoagulant activity, lysis, and cell fusion

Using 99 μs pulses, experiments were undertaken to study the effect of varying the DC breakdown voltage, over the range 2-5 kV·cm⁻¹, on the procoagulant activity, lysis, and fusion of erythrocytes in 250 mM sucrose. As with increases in the duration of the field pulses, increases in the breakdown voltage resulted in the percentage procoagulant activity rising more rapidly than the percentage haemolysis (Fig. 3.2). With an applied voltage of 5 kV·cm⁻¹, 45% of the total prothrombinase activity was exposed. However, as judged by the percentage haemolysis, only about 18% of the total prothrombinase activity was attributable to phospholipids in the inner monolayer of lysed cells. This further indicates that electrical breakdown pulses act on erythrocytes to expose PS at the cell surface.

With a breakdown voltage of 2 kV·cm⁻¹, no cell fusion occurred. As the voltage was increased, chains of fused cells of increasing length were seen. Unlike the dissimilar effects of increases in pulse length on the percentage procoagulant activity
and the percentage cell fusion (Fig. 3.1), it is apparent from Fig. 3.2 that the

\[
\text{Electric strength (kV/cm)}
\]

Figure 3.2 Human erythrocytes in a 250 mM sucrose solution, containing 1.1 mM Ca\(^{2+}\) and 10 mM histidine, were aligned in pearl chains by dielectrophoresis and subjected to three consecutive DC square wave pulses (99 \(\mu\)s), of increasing voltage (2-5 kV/cm\(^{-1}\), as described in Material and Methods. The percentage procoagulant activity (○), percentage cell fusion (Δ), and percentage haemolysis (●) were determined as described in Materials and Methods.

percentage cell fusion and the procoagulant activity increased in a comparable manner when the breakdown voltage was increased from 2.4 to 5 kV·cm\(^{-1}\).

3.3.3 Effects of hypotonic sucrose solution

Ahkong and Lucy (1986) showed that chains of up to 14 fused cells were formed when human erythrocytes in 150 mM erythritol were exposed to an electric field pulses, whereas only a few cells were fused when they were pulsed in 200 mM erythritol. NS1
mouse myeloma cells behaved in a similar manner (Brown et al., 1986). In electro-acoustic fusion of human erythrocytes, the fusion field in 170 mosM solutions was higher than the yield from cells in 272 mosM solutions (Bardsley et al., 1989). The electrofusion of mammalian cells in strongly hypo-osmolar media containing sorbitol also resulted in high yields of hybridoma cells (Schmitt & Zimmermann, 1989). The effect of varying the DC breakdown voltage, over the range 2 to 5 kV cm⁻¹, on the procoagulant activity, lysis, and fusion of erythrocytes in 150 mM sucrose solutions were therefore investigated.

![Figure 3.3 Human erythrocytes in a 150 mM sucrose solution. Containing 1.1 mM Ca²⁺ and 10 mM histidine, were aligned in pearl chains by dielectrophoresis and subjected to three consecutive DC square wave pulses (99 μs), of increasing voltage (2-5 kV cm⁻¹), as described in Material and Methods. The percentage procoagulant activity (○), percentage cell fusion (△), and percentage haemolysis (●) were determined as described in Materials and Methods.](image-url)
Although the effect of increasing the applied voltage on the prothrombinase activity of erythrocytes in 150 mM sucrose was broadly similar to that seen with the cells in 250 mM sucrose (Fig. 3.2), there was some indication that the prothrombinase activity of the pulsed cells might be slightly higher in the hypotonic solutions (Fig. 3.3). This possibility was investigated by measuring the prothrombinase activity and the haemolysis in triplicate with erythrocytes from a single donor that were subjected to pulses of 99 μs (3 kV cm⁻¹) in 150 mM and 250 mM sucrose solutions. It appears from Table 3.2 that, after allowing for the lysed cells, more procoagulant activity was exposed in the erythrocytes that were subjected to the breakdown pulses in the hypotonic sucrose solution.

<table>
<thead>
<tr>
<th>Solution</th>
<th>% prothrombinase activity</th>
<th>% haemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>150 mM sucrose</td>
<td>25.7</td>
<td>11.4</td>
</tr>
<tr>
<td></td>
<td>21.3</td>
<td>11.7</td>
</tr>
<tr>
<td></td>
<td>24.3</td>
<td>12.2</td>
</tr>
<tr>
<td></td>
<td>(mean 23.8 ± S.D. 1.8)</td>
<td>(mean 11.8 ± S.D. 0.3)</td>
</tr>
<tr>
<td>250 mM sucrose</td>
<td>15.2</td>
<td>12.2</td>
</tr>
<tr>
<td></td>
<td>14.0</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td>10.2</td>
<td>8.8</td>
</tr>
<tr>
<td></td>
<td>(mean 13.1 ± S.D. 2.1)</td>
<td>(mean 9.6 ± S.D. 1.9)</td>
</tr>
</tbody>
</table>

Table 3.2 Prothrombinase activity of human erythrocytes after exposure to electrical breakdown pulses in sucrose solutions of different osmolarity

Longer chains of fused cells were observed with erythrocytes in the hypotonic
sucrose solution than with cells in 250 mM sucrose. With erythrocytes in 150 mM sucrose solution (Fig. 3.3), as with the cells in 250 mM sucrose (Fig. 3.2), the percentage cell fusion and the percentage procoagulant activity increased in an approximately parallel manner when the breakdown voltage was increased from 2.4 to 5 kV cm\(^{-1}\).

### 3.3.4 Time course of exposure of PS

If degradation of the membrane skeleton by endogenous protease is responsible for the development of procoagulant activity in plasma membranes exposed to electrical breakdown pulses, the procoagulant activity might be expected to increase with time after delivery of the pulses. The procoagulant activity was therefore determined (in triplicate) 6 min and 36 min after the breakdown pulse with erythrocytes from a single donor that were subjected to 99 \(\mu\)s pulses (4 kV cm\(^{-1}\)) in 150 mM sucrose solution and then maintained at room temperature. Table 3.3 shows the data obtained from which it is apparent that there is little increase in surface exposure of PS between 6 min and 36 min after the field pulses; the mean value of the procoagulant activity after 36 min was only 8% greater than that after 6 min. This indicates that the exposure of PS at the cell surface was more likely to be due to a direct effect of the electric field pulses on plasma membrane structure than to secondary effects, such as the action of endogenous proteinases on the membrane skeleton.

The percentage haemolysis in Table 3.3 increased with time; the mean value of the percentage haemolysis after 36 min was 60% greater than that after 6 min. Since the haemolysis, but not the procoagulant activity of the cells increased markedly between 6 and 36 min after the field pulses, it would seem that the cells which lysed during this period also resealed almost completely.
The percentage prothrombinase activity and the percentage haemolysis were determined, in triplicate, for aligned erythrocytes from a single donor 6 min and 36 min after their exposure to three consecutive d.c. 99 μs square wave pulses (4 kV cm⁻¹) in 150 mM sucrose (containing 1.1 mM Ca²⁺ and 10 mM histidine), as described in Materials and Methods.

<table>
<thead>
<tr>
<th>Time period</th>
<th>Prothrombinase (nmol thrombin l⁻¹ min⁻¹)</th>
<th>% prothrombinase</th>
<th>% lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 min</td>
<td>10.9</td>
<td>41.5</td>
<td>19.1</td>
</tr>
<tr>
<td>36 min</td>
<td>11.6</td>
<td>44.5</td>
<td>31.5</td>
</tr>
<tr>
<td>6 min</td>
<td>13.7</td>
<td>52.4</td>
<td>18.6</td>
</tr>
<tr>
<td>36 min</td>
<td>14.0</td>
<td>53.4</td>
<td>26.6</td>
</tr>
<tr>
<td>6 min</td>
<td>14.7</td>
<td>56.1</td>
<td>15.1</td>
</tr>
<tr>
<td>36 min</td>
<td>16.4</td>
<td>62.7</td>
<td>26.4</td>
</tr>
<tr>
<td>36 min</td>
<td>(untreated cells)</td>
<td>1.4</td>
<td>5.3</td>
</tr>
<tr>
<td>Lysed cells</td>
<td></td>
<td>26.1</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.3 Prothrombinase activity of human erythrocytes at differing times after exposure to electrical breakdown pulses

3.3.5 Effect of cations on cell lysis induced by electrical breakdown

Human erythrocytes (aligned by dielectrophoresis in a solution of 250 mM sucrose and 10 mM histidine) were subjected to three electrical breakdown pulses of 99 μs (5 kV cm⁻¹) at 1-s intervals in the presence of 1.1 mM concentrations of different divalent cations (final conductivity 235 μs cm⁻¹). Table 3.4 shows that the ability of the cations to protect the cells against haemolysis by electrical breakdown decreased progressively in the order, Mn²⁺ > Ca²⁺ > Ba²⁺ > Mg²⁺ >> Zn²⁺. With 250 mM sucrose solutions of the same conductivity (235 μs cm⁻¹), but which contained Na⁺, K⁺, (2.1 mM) or Al³⁺ (0.95 mM) ions, about 99% of the cells lysed immediately on electrical breakdown.

Ca²⁺ ions are known to stimulate the resealing of lysed human erythrocytes by stimulating the contraction of osmotically induced holes in the plasma membrane. In
this respect, the potency of Ca\(^{2+}\) was found to exceed that of Mg\(^{2+}\), which greatly surpassed Na\(^{+}\), and it was suggested that Ca\(^{2+}\) facilitated the resealing of osmotically-lysed erythrocytes by stoichiometric binding to (unspecified) anionic groups on the membrane (Lieber & Steck, 1982). It, therefore, seems possible that, in the above experiments, Ca\(^{2+}\) ions facilitated membrane resealing in electrically-pulsed erythrocytes by binding to molecules of phosphatidylserine that were exposed on the cell surface by electrical breakdown.

<table>
<thead>
<tr>
<th>Divalent cation</th>
<th>% cell lysis ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn(^{2+})</td>
<td>14.4 ± 1.4 (3)</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>15.1 ± 0.6 (4)</td>
</tr>
<tr>
<td>Ba(^{2+})</td>
<td>21.5 ± 2.9 (3)</td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>24.9 ± 3.2 (4)</td>
</tr>
<tr>
<td>Zn(^{2+})</td>
<td>44.1 ± 2.4 (3)</td>
</tr>
</tbody>
</table>

Table 3.4 Effects of divalent cations on the lysis of human erythrocytes subjected to electrical breakdown pulses

3.3.6 Effects of cations on cell fusion induced by electrical breakdown

The number of fused cells in the pearl chains, which were formed when human erythrocytes were exposed to breakdown pulses as above, was greatest in the presence of Mn\(^{2+}\), and decreased progressively with Ba\(^{2+}\), Ca\(^{2+}\), and Mg\(^{2+}\) (Table 3.5). It is also apparent from Table 3.5 that this decreasing effectiveness is inversely related to previously reported data on (i) the threshold concentrations of cations required to induce the fusion of 500 Å diameter vesicles of phosphatidylserine; (ii) the concentrations of
cations needed to increase the interfacial tension in monolayer membranes of phosphatidylserine by 6-8 dyne·cm\(^{-1}\) (Ohki, 1988). Our observations therefore support the concept that the surface exposure of acidic phospholipids plays an important role in the fusion of human erythrocytes induced by electrical breakdown.

<table>
<thead>
<tr>
<th>Divalent cation</th>
<th>No. of cells in chains of electrofused cells with 1.1 mM cation (\pm) S.D.</th>
<th>Threshold concentration for the fusion of vesicles of phosphatidylserine (mM)</th>
<th>Concentration for increase in tension of 7-8 dyne cm(^{-1}) in monolayers of phosphatidylserine (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn(^{2+})</td>
<td>4.48 ± 0.28 (4)</td>
<td>0.85</td>
<td>0.7</td>
</tr>
<tr>
<td>Ba(^{2+})</td>
<td>4.26 ± 0.22 (3)</td>
<td>–</td>
<td>0.8</td>
</tr>
<tr>
<td>Zn(^{2+})</td>
<td>3.72 ± 0.14 (3)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>3.50 ± 0.07 (3)</td>
<td>1.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>2.28 ± 0.04 (3)</td>
<td>6.0</td>
<td>6.0</td>
</tr>
</tbody>
</table>

**Table 3.5** Effects of divalent cations on the fusion of human erythrocytes induced by electrical breakdown, and on the properties of model membrane systems of PS. The threshold concentrations of divalent cations for the fusion of 500 Å diameter vesicles of PS were derived by interpolation from Fig.2 in Ohki, 1988. The concentrations of cations found to increase the interfacial tension of monolayers of PS by 7-8 dyne/cm are from Table I in Ohki, 1988.

Erythrocytes that were subjected to electrical breakdown in 250 mM sucrose solutions which contained Na\(^{+}\), K\(^{+}\), (2.1 mM) or Al\(^{3+}\) (0.95 mM) ions (conductivity 235 \(\mu\)s·cm\(^{-1}\)) were unable to form pearl chains under current conditions. In this connection it is relevant that up to 1 M concentrations of Na\(^{+}\) and K\(^{+}\) failed to induce the fusion of small unilamellar vesicles of phosphatidylserine, or to increase the interfacial tension in monolayer membranes of phosphatidylserine (Ohki, 1988).

The data presented here show that, apart from the behaviour of erythrocytes with
Zn\(^{2+}\), there is a general relationship between protection against haemolysis by cations and the formation of chains of fused cells in the presence of these ions. Thus, Mn\(^{2+}\) was most effective in protecting the cells against electrically-induced lysis and in facilitating the formation of chains of erythrocytes, while Mg\(^{2+}\) was the least effective on both counts. Ca\(^{2+}\) and Ba\(^{2+}\) were intermediate between Mn\(^{2+}\) and Mg\(^{2+}\), although not in the same order. Na\(^{+}\), K\(^{+}\), and Al\(^{3+}\), gave virtually no protection against haemolysis, and the cells were not fused by electrical breakdown pulses.

Zn\(^{2+}\) has been reported to induce the fusion of vesicles composed of dipalmitoyl phosphatidylcholine (DPPC)/PS (50%/50% or 60%/40%) at considerably lower concentrations than Ca\(^{2+}\) (Barfield & Bevan, 1985). It might therefore be expected that, as with Mn\(^{2+}\), relatively long chains of fused cells would be formed by electrical breakdown in the presence of Zn\(^{2+}\). However, replacement of 1.1 mM Ca\(^{2+}\) in the fusion medium by 1.1 mM Zn\(^{2+}\) had little effect on the lengths of the chains of fused cells. As discussed below, the swelling behaviour of erythrocytes subjected to electrical breakdown in the presence of Zn\(^{2+}\) was also found to be anomalous.

3.3.7 Ca\(^{2+}\) ions, osmotic swelling, and the formation of giant cells

In early work, it was reported that the addition of a medium containing 1 mM Ca\(^{2+}\) ions (but otherwise of low conductivity) was often required about 1 min after the breakdown pulse, or else the cells did not become spherical (Arnold & Zimmermann, 1984). However, we obtained a quite different result when erythrocytes, aligned by dielectrophoresis, were subjected to a single electrical breakdown pulse of 20 \(\mu\)s (3.5 kV cm\(^{-1}\)) in hypotonic solutions of 150-200 mM erythritol (containing 5 mM histidine) without either Ca\(^{2+}\) or commercial pronase (which stabilises cells against the AC field and DC pulses at least partly because it contains Ca\(^{2+}\) (Zimmermann et al., 1981;
Ohno-Shosaku & Okada, 1985). (The comparatively high AC field strength used to align the cells by dielectrophoresis was applied as described in Materials and Methods.) Under these conditions, the fused cells swelled rapidly, giant cells formed in 10-30 s., and cell lysis was complete within a few minutes. In a separate experiment, the addition of small quantities of distilled water to erythrocytes after their exposure to breakdown pulses in a sucrose-containing medium caused them to round-up into giant cells. These findings indicated that cell swelling, rather than Ca^{2+} ions, facilitates the rounding-up of electrically-fused erythrocytes into giant cells.

Erythrocytes lose ions and shrink immediately following electrical breakdown. The permeabilised cells then swell osmotically because of their impermeable haemoglobin, and the rate of swelling is decreased by the presence of molecules of increasing size (Kinosita & Tsong, 1977a). In order to investigate further the role of osmotic swelling in the rounding-up of fused erythrocytes into giant cells, erythrocytes were therefore subjected to electrical breakdown in solutions of carbohydrate molecules of increasing size (which also contained 1.1 mM Ca^{2+}). Sugars ranging from the 4-carbon sugar, erythritol (radius approx. 3.1 Å), to the 12-carbon sugar, sucrose (radius approx. 4.4 Å) were investigated in current experiment. 5 min after the pulses, cells in 250 mM erythritol media were much less shrunken than cells in 250 mM sucrose media. After 45 min with erythritol, the cells were extensively swollen, a high proportion of the fused cells were rounding-up into giant cells, and some spherical giant cells were present (Fig. 3.4A). By comparison, the rounding-up was considerably less advanced after 60 min with the 5-carbon sugar, ribitol (adonitol), and evidence of cell shrinkage was still apparent (Fig. 3.4B). After 90 min, cells with ribitol (Fig. 3.4C) were comparable with the 45 min preparations of cells with erythritol (Fig. 3.4A), and
Figure 3.4 Panels A-G show human erythrocytes at 25°C, aligned by dielectrophoresis in 250 mM solutions of sugars (containing 10 mM histidine and 1.1 mM Ca^{2+}), that were subjected to three electrical breakdown pulses of 99 μs (5 kV/cm) at 1-s intervals. Panel A (with erythritol, 45 min after breakdown): most of the cells were swollen, fused cells were rounding-up into giant cells (long arrow), spherical giant cells were present (short arrow), but many cells exhibited evidence of shrinkage. Panel C (90 min with ribitol), the cells were comparable to 45 min preparations with erythritol. Panel D (90 min with mannitol), the rounding-up process was less advanced than after 90 min with ribitol. Panel E (2 h with sucrose) shows persistent cell shrinkage. Panel F (5 h with sucrose) shows occasional giant cell formation (arrow) among the shrunken cells. Panel G shows that cell swelling, rounding-up and giant cell formation were markedly inhibited after 60 min by the presence of 30 mM dextran ($M_r$ 4000-6000) in the erythritol medium. Panel H shows erythrocytes, subjected to electrical breakdown as for panels A-G, except that 1.1 mM Zn^{2+} was present instead of 1.1 mM Ca^{2+}. Extensive cell rounding occurred, and a giant cell was present (arrow) only 15 min after breakdown in sucrose media that contained Zn^{2+} ions. Magnification: ×440. Each experiment was done at least three times, and the micrographs shown are representative of the observations made.
the rounding-up was more advanced than in 90 min preparations with the 6-carbon sugar, mannitol (Fig. 3.4D). Cells in 250 mM sucrose solutions rounded-up extremely slowly, and most of the cells remained shrunken after 2 h (Fig. 3.4E). Occasional giant cells were observed 5 h after breakdown, but shrunken erythrocytes were in a majority (Fig. 3.4F).

When a non-permeable dextran ($M_\text{w} 4000-6000$) (30 mM) was present in the 250 mM erythritol medium, cell swelling, rounding-up, and giant cell formation were markedly inhibited by comparison with cells in erythritol medium (280 mM) without dextran. Thus, after 60 min with the added dextran, the erythrocytes were very shrunken, and no cell rounding or giant-cell formation was observed (Fig. 3.4G). Similarly, cells in 250 mM erythritol media which contained 30 mM poly(ethylene glycol) 3350, or 3 mM inulin ($M_\text{w} 5000$), rounded-up more slowly than cells in the presence of erythritol alone. If these poly(ethylene glycol)- or inulin-containing media were subsequently replaced by a medium containing erythritol alone, cell swelling and giant cell formation proceeded unhindered. These several observations showed that the rounding-up of fused human erythrocytes into giant cells depends on osmotic swelling and parallels the well-documented swelling properties of erythrocytes which have been permeabilised by electrical breakdown (Kinosita & Tsong, 1977b).

3.3.8 Osmotic swelling, hemi-fusion, and cell fusion

With relatively short breakdown pulses (20 $\mu$s) and low applied DC voltage (2.7 kV cm$^{-1}$), human erythrocytes exhibit hemi-fusion, i.e. membrane fusion without cytoplasmic fusion (see Chapter Two). However, in the present experiments, the relatively long pulses (99 $\mu$s) and high breakdown voltage (5 kV cm$^{-1}$) would have resulted in the majority of the pulsed cells being completely fused. Table 3.6 shows that
Human erythrocytes, aligned by dielectrophoresis in 250 mM solutions of different sugars, containing 10 mM histidine and 1.1 mM Ca^{2+}, were subjected to three electrical breakdown pulses of 99 µs (5 kV/cm) at 1-s intervals. The percentage of cell fusion was determined, for the numbers of separate experiments shown in brackets, as described in Materials and Methods.

<table>
<thead>
<tr>
<th>Non-ionic solute</th>
<th>%cell fusion ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythritol</td>
<td>57.9±1.7 (3)</td>
</tr>
<tr>
<td>Ribitol</td>
<td>53.6±3.5 (3)</td>
</tr>
<tr>
<td>Mannitol</td>
<td>53.1±2.9 (3)</td>
</tr>
<tr>
<td>Sucrose</td>
<td>50.8±2.8 (3)</td>
</tr>
</tbody>
</table>

Table 3.6 Effects of sugars on the cytoplasmic fusion of human erythrocytes induced by electrical breakdown pulses

the percentage cell fusion for erythrocytes in 250 mM solutions of different sugars decreased only slightly in the following order: erythritol > ribitol (adonitol) ≥ mannitol > sucrose. It would therefore seem that cell fusion, unlike the formation of giant cells, is not markedly affected by the size of the sugar present. The slightly higher percentage cell fusion observed with erythritol, by comparison with the other three sugars (Table 3.6), may nevertheless have resulted from osmotic swelling in erythrocytes media that induced any hemi-fused cells present to fuse completely. This interpretation is supported by an earlier finding that delayed cell fusion occurred when electrically-permeabilised erythrocytes were allowed to swell in 200 mM erythritol (Lucy, 1986). Hemi-fused human erythrocytes, produced by electrical breakdown, also gave rise to completely fused cells on heating to about 50°C (Chapter Two).

3.3.9 Zn^{2+} ions and the formation of giant cells

Zn^{2+} apparently inhibited the immediate loss of ions that occurred when erythrocytes were subjected to electrical breakdown in low ionic strength media, since the cells failed to shrink immediately after breakdown in the presence of 1.1 mM Zn^{2+}

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and they subsequently swelled very rapidly. Thus, large numbers of giant cells were present in only 10-15 min (Fig. 3.4H). This rapid cell swelling was consistent with the high level of lysis observed with erythrocytes subjected to breakdown in the presence of Zn$^{2+}$ (Table 3.4). By contrast, the addition of Zn$^{2+}$ to (a Ca$^{2+}$ - containing medium) after electrical breakdown did not accelerate cell swelling and the formation of giant cells, indicating that Zn$^{2+}$ did not increase the size of pores through which sucrose enters electropermeabilised erythrocytes.

Cadmium and cobalt ions had similar effects to Zn$^{2+}$, but attempts to mimic the action of Zn$^{2+}$ with 1 mM concentrations of inhibitors of voltage-dependent K$^+$ channels (4-aminopyridine and tetraethylammonium chloride) were unsuccessful.

3.4 Discussion

The rate of conversion of prothrombin to thrombin by the enzyme complex factor Xa-factor Va, which were employed in current experiments, has been used quite extensively as a convenient, sensitive, and semi-quantitative way of monitoring the surface exposure of PS in the plasma membranes of platelets (Bevers et al., 1989) and erythrocytes (Middelkoop et al., 1988). In the latter work, it was shown that a combination of sickling and ATP depletion resulted in a time-dependent increase in PS in the outer membrane leaflet of human erythrocytes, as measured by phospholipase A$_2$ accessibility and by the prothrombinase assay.

Pronase was included in the fusion medium in previous work on the electrofusion of erythrocytes (Chapter Two), since an early investigation showed that pronase stabilises cells against lysis caused by electrical breakdown (Zimmermann et
This appears to result from proteolytic action on the cells and the presence of contaminating Ca\(^{2+}\) in commercial preparations of pronase (Ohno-Shosaku & Okada, 1985). Since Ca\(^{2+}\) ions alone substantially protect human erythrocytes against electrical breakdown, and it was desirable to avoid the proteolytic effects of pronase in the present experiments, a pronase-free medium that contained 1.1 mM Ca\(^{2+}\) was used in the work reported here. Cell fusion occurred similarly in the presence of Mg\(^{2+}\) (1.1 mM). However, Mg\(^{2+}\) was less effective than Ca\(^{2+}\) in protecting against cell lysis, and this precluded experiments on possible changes in phospholipid asymmetry being investigated in the presence of Mg\(^{2+}\).

Earlier analyses of changes in phospholipid asymmetry associated with electric breakdown were made on ghosts after they had been incubated for 60 min (Dressler et al., 1983). It is therefore possible that the observed changes may have arisen from secondary effects, such as the action of endogenous proteinases, rather than from a direct effect of the electric field pulses on membrane structure. The analyses of current work commenced only 6 min after exposure of the cells to breakdown pulses, and there was only a small increase in procoagulant activity during the following 30 min. Furthermore, the measurements were on cell suspensions in which the percentage haemolysis was less than 20% (with the 10 \(\mu\)s pulses in Fig. 3.1, haemolysis was only 10%). Secondary effects cannot be completely excluded, however, as we were unable to determine whether changes in phospholipid asymmetry occurred during the first 6 min. after the pulses.

In the experiments partial resealing of lysed erythrocytes prior to commencing the prothrombinase assays, may have resulted in PS in the inner monolayer regaining their inaccessibility. The contribution to the procoagulant activity that was attributed to
PS in the inner monolayer, as judged from the percentage haemolysis, could therefore have been overestimated (particularly as the extent of haemolysis was determined after the prothrombinase assays were completed). Consequently, in the experiment of Fig. 3.1, there may have been an almost complete loss of asymmetry in the distribution of PS in erythrocytes subjected to breakdown pulses longer than about 20 µs.

In the plasma membranes of eukaryotes, aminophospholipid asymmetry appears to be due to the ATP-dependent aminophospholipid translocase activity that counterbalances the spontaneous lipid randomization by flip-flop (Devaux, 1991). Interactions of the acidic phospholipids with the membrane skeleton may also contribute to the maintenance of phospholipid asymmetry in erythrocytes (Devaux, 1991), in which case damage to the membrane skeleton caused by field pulses might be expected to give rise to a loss of phospholipid asymmetry. Voltage-dependent blebbing of the plasma membrane that was inhibited by increasing the tonicity of the medium has been observed by light microscopy with several cell types exposed to electric field pulses, and it has been suggested that this phenomenon may have resulted from a local rupture of the cytoskeleton (Gass & Chernomordik, 1990). Although no blebbing was observed with human erythrocytes subjected to electric field pulses, it may be relevant that the loss of phospholipid asymmetry in human erythrocytes which occurs when they are treated with Ca²⁺ and ionophore A23187 is apparently associated with shedding of vesicles from the plasma membrane (Comfurius et al., 1990).

It is relevant to present findings that the application of electrical pulses to yeast cells has been observed to induce an increase in absorption of the cationic dye, 9-aminoacridine. This may have been due to the appearance of additional electronegative groups on the outer side of the plasma membrane (Tomov & Tsoneva, 1989). A study
made with $^{31}$P-NMR of electropermeabilised Chinese hamster ovary cells also showed that the polar head groups of about 70% of plasma membrane phospholipids were in a new configuration in the permeabilised cells, and it has been proposed that this leads to a decrease in hydration repulsion forces between the treated cells that facilitates cell fusion (Lopez et al., 1988; Rols & Teissié, 1990).

It was shown that breakdown pulses of 2-5 ms caused a marked loss of phospholipid asymmetry, but no cell fusion was observed unless the pulse length was at least 20 ms (Fig. 3.1). A loss of phospholipid asymmetry in the plasma membrane is therefore not sufficient to induce fusion. However, human erythrocytes that have apparently lost their phospholipid asymmetry have been found to exhibit an increased susceptibility to fusion induced by poly(ethylene glycol) 6000 (Tullius, 1989). Surface exposure of PS also occurs in the fusion of human erythrocytes induced by protocols that involves cell swelling (Baldwin, 1990). Since approximately parallel increases in the surface exposure of PS and cell fusion with breakdown pulses of 99 ms and increasing voltage were observed, it seems probable that exposure of PS at the cell surface facilitates the fusion process but that fusion cannot occur in the absence of other events, e.g. the development of structural defects in the phospholipid bilayer.

The primary work involved in bringing phospholipid bilayers into close apposition is necessary to dehydrate their polar head groups. Hydration repulsion between bilayers of anionic phospholipids is readily overcome, however, by the addition of Ca$^{2+}$ ions to the aqueous phase. Most of water between the two membrane surfaces is then displaced, since the Ca$^{2+}$ ions bind to the polar groups of the anionic phospholipids, and the membrane collapse together (Rand & Parsegian, 1989). Movement of PS from the inner to the outer monolayer of the plasma membrane of
erythrocytes in the presence of Ca$^{2+}$ ions would thus be expected to decrease the forces of hydration repulsion between approaching cells, and it has been suggested that the fusogenic state of the plasma membrane that results from the exposure of cells to electrical field pulses may involve a loss of phospholipid asymmetry (Tsong, 1990). The present observations are consistent with this suggestion. A localised, surface exposure of acidic phospholipid may therefore contribute to the ‘long-lived fusogenic state’ (Sowers, 1986) and the ‘transient permeant structures’ (Teissié & Rols, 1986) that enable cell fusion to occur when contact between cells is established after they have been subjected to field pulses. However, the long-lived fusogenic state has a much shorter life-time (Sowers, 1987) than the change in phospholipid asymmetry observed in current experiments. This would be anticipated if the surface exposure of acidic phospholipids is only one of several factors that contribute to the fusogenic state.

The transient pores induced by electroporation in the membranes of erythrocytes (Chang & Reese, 1990), the transition point defects which precede the electro-fusion of erythrocytes (Stenger & Hui, 1986), and the similar but more stable defects which are associated with the electrically-induced, hemi-fusion of these cells (Chapter Two), may correspond physically to sites at which phospholipid asymmetry has been lost. A surface exposure of PS at localised sites in the plasma membranes of erythrocytes as a consequence of electrical breakdown would additionally be expected to facilitate cell fusion in view of the fact that the addition of sufficient PS to liposomes composed of PC leads to membrane fusion in the presence of Ca$^{2+}$ ions (Düzgüneş et al., 1981).

Mn$^{2+}$ was found to be most effective, and Mg$^{2+}$ least effective, in facilitating the formation of pearl chains of fused cells on electrical breakdown in the present work. Ca$^{2+}$, although less effective than Mn$^{2+}$, was far more effective than Mg$^{2+}$. These
relationships are consistent with the idea that exposure of PS at the cell surface plays an important role in electrically-induced cell fusion, since these three ions have comparable activities in the fusion of vesicles of PS (Table 3.5) (Ohki, 1988).

Mn$^{2+}$ was also most effective, and Mg$^{2+}$ least effective, in protecting the cells against lysis resulting from electrical breakdown. Again, Ca$^{2+}$ was less effective than Mn$^{2+}$, but far more effective than Mg$^{2+}$ (Table 3.4). So far, no molecular explanation appears to have been put forward for the ability of Mn$^{2+}$ and Ca$^{2+}$ to facilitate cell fusion and inhibit lysis simultaneously (Zakai et al., 1974; Ahkong & Lucy, 1988; Baldwin et al., 1990). From the present experiments, it could be proposed that this relationship results from the ability of these cations to interact with PS, at least with erythrocytes that are induced to fuse by osmotic swelling, or by electrical breakdown. Thus the ability of Ca$^{2+}$ to form a bridge between PS that is exposed on the surfaces of adjacent erythrocytes (Baldwin et al., 1990) will facilitate the close approach of closely apposed membranes that is necessary for cell fusion. Since Ca$^{2+}$ is thought to facilitate the resealing of lysed human erythrocytes by stoichiometric binding to anionic groups on the membrane (Lieber & Steck, 1982), it is suggested that Ca$^{2+}$ additionally interacts with PS at the cell surface to facilitate membrane resealing following electrical breakdown, thus stabilising the cells and allowing them to fuse. (Ca$^{2+}$ ions that enter the permeabilised cells may similarly facilitate resealing by binding to PS on the cytoplasmic surface). The present findings also give circumstantial support to the concept that changes in phospholipid asymmetry may be important in physiologically-ocurring instances of biomembrane fusion, such as the fusion of myoblasts (Sessions & Horwitz, 1981) and the exocytosis of chromaffin granules (Zachowski et al., 1989).

It is relevant that Papahadjopoulos (1968) showed that monolayers of PS at an
air-water interface interact strongly with low concentrations of divalent cations (10^4 to 10^3 M) in the presence of physiological concentrations of univalent salts, and that the interactions are accompanied by a decrease in surface pressure (condensation). He suggested a structure for the complex of PS with Ca^{2+}, in which each Ca^{2+} ion interacts (via six coordination bonds) with four phospholipid molecules to give a linear, polymeric arrangement.

Following electrically-induced cell fusion, erythrocytes round-up into giant cells. It has previously been proposed that Ca^{2+} rather than osmotic forces, accelerate the rounding-up of cells after fusion has been induced by electrical breakdown (Zimmermann, 1982). By contrast, the importance of colloid osmotic swelling in the behaviour of human erythrocytes, following their permeabilisation by electrical breakdown, has been well documented (Kinosita & Tsong, 1977a, 1977b; Tsong, 1990; Schwister & Deuticke, 1985; Deuticke et al., 1991). Thus, DC pulses, of approx. 4 kV·cm^{-1} for 5-120 μs, give rise to defects in the plasma membrane, which have the properties of aqueous holes with definable radii and selectivities, and which permit the passage of ions and small molecules but not haemoglobin. The permeabilised cells swell osmotically because of their impermeable haemoglobin, and they lyse when the cell volume approaches 155% of the normal value. The rates of swelling and of haemolysis are decreased by the presence of molecules of increasing size. Added oligosaccharides or small proteins, which are larger than the size of the electropores, retard haemolysis indefinitely because they osmotically balance the impermeable haemoglobin in the cytoplasm (Kinosita & Tsong, 1977b). At 37°C, resealing of the pores occurs within minutes to hours. Thus, for pores that permit permeation of Rb^+ but not sucrose, resealing take about 20 min. Pores that admit sucrose initially, as in the present
experiments, need about 20 h to reseal completely (Tsong, 1990).

It is clear from the present observations on the electrical breakdown of erythrocytes in the presence of variously permeable sugars that the rounding-up of the electrofused cells into giant cells parallels the well-documented swelling of individual erythrocytes. Thus, immediately following exposure to breakdown pulses, human erythrocytes rapidly lose ions and shrink (Kinosita & Tsong, 1977a). Erythritol can enter the pulsed cells, via the electropores, more rapidly than sucrose (Kinosita & Tsong, 1977b). This allows the formation of giant cells, and lysis of the electrofused cells, to occur more rapidly in the presence of erythritol than in the presence of sucrose. However, electropores that are sufficiently large to admit sucrose remain open for many hours. As a result, some fused erythrocytes in a sucrose-containing medium round-up in 5 h. The relationship between the rounding-up of electrofused cells, giant cell formation, and the swelling of individual electropermeabilised cells is further demonstrated by the fact that the presence of large impermeable molecules in the medium inhibited cell rounding and the formation of giant cells.

The observations on the role of cell swelling in the formation of giant cells following electrical breakdown also parallel the well-established importance of osmotically-induced cell swelling in the formation of giant erythrocytes by haemolytic Sendai virus particles. Fusion of Sendai virus particles with the plasma membrane of cells makes the membrane permeable to low molecular weight compounds and ions, and this leads to cell swelling (Poste & Pasternak, 1978; Knutton et al., 1976). At least for fusion that involves a virion acting as a bridge between two cells, the swelling is responsible for the rounding up of already fused cells (Pasternak, 1984). Thus, it was shown that the formation of giant cells was prevented when osmotic swelling was
inhibited by hypertonic media (Knutton & Pasternak, 1979; Imprain et al., 1980). Furthermore, when early-harvested (non-haemolytic and non-leaky) Sendai virions were used, giant cells were not formed (Knutton, 1979; Wyke et al., 1980). That cell fusion nevertheless occurred under these circumstances was demonstrated by showing that the treated erythrocytes enlarged into giant cells on subsequent exposure to a hypotonic medium (Knutton & Bachi, 1980). It was therefore concluded that osmotic swelling appears to be the driving force that results in cells, in which membrane-fusion sites are present, being able to expand into poly-erythrocytes. Work on the fusion by Sendai virus of human erythrocyte ghosts, which were prepared with or without sequestered macromolecules, such as bovine serum albumin or dextran, has also shown that colloidal osmotic swelling is responsible for the rounding-up of virally-fused ghosts (Sekiguchi et al., 1981). The present findings on the role of osmotic swelling in the formation of giant cells from human erythrocytes that are exposed to electrical breakdown pulses are, therefore, fully consistent not only with the swelling properties of erythrocytes which have been permeabilised by electrical breakdown, but also with the formation of giant erythrocytes and poly-ghosts resulting from the action of the Sendai virus on intact and lysed erythrocytes, respectively.

By comparison with their responses to electrical breakdown in the presence of Mn$^{2+}$, Ca$^{2+}$, Ba$^{2+}$, Mg$^{2+}$, Na$^{+}$, K$^{+}$, and Al$^{3+}$, human erythrocytes behaved anomalously in the presence of Zn$^{2+}$ with regard to lysis, fusion, and cell swelling in the current experiments. In relation to the ability of Zn$^{2+}$ to inhibit the rapid cell shrinkage that otherwise ensued when erythrocytes were subjected to electrical breakdown in media of low-ionic strength, it may be relevant that Zn$^{2+}$ is well known to inhibit the leakage of cations, negatively-charged, and neutral molecules from cells.
that have suffered plasma membrane damage by haemolytic virus, toxins, complement, and other cytotoxic agents (Bashford et al., 1986). Zn$^{2+}$ is some 10-fold more effective than Ca$^{2+}$ in this respect. It is also interesting that recent work on Na$^{+}$ channels in plasma membranes from mammalian heart ventricular muscle, which are blocked by Zn$^{2+}$ with a ~ 100-fold higher affinity than other Na$^{+}$ channel subtypes, has indicated that Zn$^{2+}$ appears to bind to a site that contains one or more cysteine sulphhydryl groups (Schild & Moczydlowski, 1991). Conceivably then, Zn$^{2+}$ may inhibit the immediate loss of ions from erythrocytes that are subjected to electrical breakdown in low-ionic-strength media by interacting with cysteine groups in membrane proteins.

Summary

The procoagulant activity of human erythrocytes, which provides a measure of the translocation of phosphatidylserine (PS) from the inner to the outer monolayer of the plasma membrane, has been compared with the percentage cell fusion in experiments on the effects of electrical breakdown pulses under differing experimental conditions. After treatment with breakdown pulses of 20 $\mu$s or longer (5 kV cm$^{-1}$), the plasma membranes of erythrocytes in 250 mM sucrose exhibited an almost complete loss of asymmetry with respect to PS. As the breakdown voltage was increased from 2 to 5 kV cm$^{-1}$ (with breakdown pulses of 99 $\mu$s), the surface exposure of PS and cell fusion increased approximately in parallel. Furthermore, with 99 $\mu$s pulses and a voltage of 3 kV cm$^{-1}$, a decrease in the osmolarity from 250 to 150 mM of the sucrose medium was accompanied by an increase in both the surface exposure of PS and the extent of cell fusion. Breakdown pulses of 2-5 $\mu$s were sufficient to cause a marked loss of asymmetry, but no cell fusion was observed unless the pulse length was at least 20
μs. Kinetic experiments indicated that exposure of PS at the cell surface was more likely to be due to a direct effect of the electric field pulses on plasma membrane structure than to secondary effects, such as the action of endogenous proteinases on the membrane skeleton. It seems possible that a localised, surface exposure of PS may contribute to the 'long-lived fusogenic state' (Sowers, 1986) and the 'transient permeant structures' (Teissié & Rols, 1986) that enable cell fusion to occur when contact between cells is established after they have been subjected to field pulses. These observations also provide circumstantial support to the concept that changes in phospholipid asymmetry may be important in physiologically-occurring instances of biomembrane fusion.

In the light of the finding of the relationships between the surface exposure of PS and cell fusion in erythrocytes subjected to electrical breakdown, factors that govern cell lysis, cell fusion, and the formation of giant cells induced by electrical breakdown with human erythrocytes in media of low ionic strength were investigated and characterised. Divalent cations (1.1 mM) protected the cells against haemolysis in the order Mn$$^{2+}$$ > Ca$$^{2+}$$ > Ba$$^{2+}$$ > Mg$$^{2+}$$ > > Zn$$^{2+}$$, whereas about 99% of the cells lysed immediately on electrical breakdown in the presence of Na$$^+$$ or K$$^+$$ (2.1 mM), or Al$$^{3+}$$ (0.95 mM). The lengths of pearl chains of fused erythrocytes formed was similarly greatest with Mn$$^{2+}$$ and decreased progressively with Ba$$^{2+}$$, Zn$$^{2+}$$, Ca$$^{2+}$$ and Mg$$^{2+}$$. No cell fusion occurred with Na$$^+$$, K$$^+$$ or Al$$^{3+}$$. It is suggested that interactions with phosphatidylserine, which is exposed at the cell surface by electrical breakdown, may enable Mn$$^{2+}$$, Ba$$^{2+}$$ and Ca$$^{2+}$$ ions to inhibit cell lysis (via membrane resealing) and facilitate cell fusion. Following electrically-induced cell fusion, erythrocytes round-up into giant cells. It has previously been proposed that Ca$$^{2+}$$ ions accelerate the rounding-
up process. However, data are presented which show that, as with erythrocytes treated with Sendai virus, the formation of rounded, giant cells following cell fusion depends on the osmotic swelling properties of permeabilised erythrocytes. Osmotic swelling may also have induced any hemi-fused cells present to fuse completely. Zn\(^{2+}\) ions anomalously enabled erythrocytes to round-up very rapidly into giant cells following electrical breakdown. This phenomenon may result from an interaction of Zn\(^{2+}\) ions with cysteine groups in membrane proteins, which decreases the immediate loss of ions that occurs when erythrocytes are subjected to electrical breakdown in low-ionic-strength media.
Chapter Four

Membrane fusion induced by peptides corresponding to the N-terminus of HIV-1 virus gp41 proteins and influenza virus hemagglutinin; their orientation and secondary structure in lipid membranes studied by Fourier transform infrared spectroscopy.

4.1 Introduction

4.1.1 Influenza virus and HIV virus induced membrane fusion

4.1.1.1 Influenza virus

Influenza viruses are enveloped, negative-strand RNA viruses belonging to the orthomyxovirus family. Influenza viruses generally infect epithelial cells in the respiratory tract in humans, other mammals, and birds. Influenza epidemics continue to be a major cause of death and suffering for humans, and pose a threat to livestock.

Influenza virions range in diameter from 80 to 120 nm, and are present in a variety of shapes from spherical to oblong. The viral envelope contains three proteins: HA (hemagglutinin), NA (neuraminidase), and M2. HA is a homotrimer, while NA and M2 are homotetramers. HA and NA protrude as spikes from the viral membrane.

HA is the only influenza protein required for fusion activity (White et al., 1982a). To enter a host cell, an influenza virus particle attaches to the cell surface through the HA to sialic acid containing glycoproteins or glycolipids (Wiley & Skehel, 1987). This depends on multiple HA-receptor interactions. The bound virus particles are then internalized by the cell via receptor-mediated endocytosis, usually in clathrin-coated vesicles (Matlin et al., 1981). Within minutes of endocytic uptake, the virus particles are delivered to early endosomes, in which the pH is about 6 (Schmid et al., 1989). As the virus particles continue their journey within the endocytic pathway, the

*(Stegmann & Helenius, 1993)
pH decreases gradually (Mellman et al., 1986). The threshold pH for HA-mediated membrane fusion varies from 5 to 6 between virus strains. Eventually, a pH low enough to trigger the conformational change is reached and membrane fusion occurs. The genome content of the virus particle is released from the endosome. This usually occurs 5 to 30 min after endocytic uptake (Martin & Helenius, 1991).

The bromelain-released, soluble ectodomain of influenza envelope glycoprotein hemagglutinin (neutral form of BHA) is the only membrane fusion protein whose X-ray crystallographic structure has been solved (Wilson et al., 1981). The HA spike protrudes about 135 Å from the viral surface. Each monomer consists of two disulfide-linked subunits, HA1 (47 kDa) and HA2 (28 kDa), which are generated from a single polypeptide chain, HA0 (85 kDa), by post-translational cleavage involving a host cell protease. The N terminus of HA2 generated from the cleavage appears crucial for the expression of fusion activity (Klenk et al., 1975; Lazarowitz & Choppin, 1975). Site-specific mutants within the N terminus of HA2 severely affect the fusion activity of HA (Gething et al., 1986). The N terminus of HA2 contains a highly conserved hydrophobic stretch of amino acids which is often referred to as the fusion peptide (Gething et al., 1986; Lear & DeGrado, 1987).

Most of HA1 forms the globular head region which contains the sialic acid binding site (Sauter et al., 1989). The N-terminus of HA2 (fusion peptide) is followed by a short antiparallel β sheet. The β sheet is connected to a short α helix. The short α helix is connected to a long α helix by an extended loop region. The long α helix interacts with the corresponding long α helices from two other HA2 polypeptides in the HA trimer to form a 105 Å long three stranded coiled coil. The coiled coil of three α helices and the remainder of HA1 form the fibrous stem of the HA trimer. The three
shorter α helices are displayed on the outside of the coiled coil (Wilson et al., 1981). The C terminus of HA2 subunit contains the transmembrane domain of HA.

In the neutral pH structure of HA, the three fusion peptides, one per monomer, are buried in the fibrous stem of the molecule, about 35 Å away from the viral membrane and 100 Å away from the distal tips of HA1 globular head domains. The fusion peptides are tightly tucked into the subunit interface by a network of hydrogen bonds (Wilson et al., 1981).

At low pH, the fusion peptides are exposed (White & Wilson, 1987) and changes occur at the distal tips of the head domains, probably reflecting their partial separation (Kemble et al., 1992). The globular heads dissociate substantially from one another, which are revealed by epitope exposure (White & Wilson, 1987) and by electron microscopy (Ruigrok et al., 1986; Stegmann et al., 1987; Puri et al., 1990). If intersubunit disulfide bonds in the membrane-distal region of the influenza hemagglutinin are introduced, exposure of the fusion peptides and fusion are severely impaired (Godley et al., 1992; Kemble et al., 1992).

However, recent observations suggest that the fusion-active form of HA is different from the fully dissociated trimer. It corresponds to a transient conformational intermediate which became apparent when the fusion of the virus with liposomes and erythrocyte membranes were analyzed at 0°C (Stegmann et al., 1990). At this temperature, fusion was still quite efficient compared to fusion at 37°C. Exposure of the fusion peptides was found to take place within 15 s after acidification, however, no detectable alterations were seen at the top of the trimer (Stegmann et al., 1990). This fusogenic conformational intermediate of HA was found to be similar to the intermediate found in BHA by White and Wilson (1987). The absence of detectable
changes in the top domains does not necessarily mean that they do not undergo any alteration at all. It is likely that they move at least transiently with respect to each other and permit the extrusion of fusion peptides from the trimer interfaces (Stegmann & Helenius, 1993).

HA is the best-characterised membrane fusion protein so far and extensive studies of HA-related membrane fusion have been done. Many reviews have been produced in this area (Wiley & Skehel, 1987; Wharton, 1987; Stegmann et al., 1989; White, 1990, 1992; Doms et al., 1990; Stegmann & Helenius, 1993; Clague et al., 1993; Wilschut & Bron, 1993; Bentz et al., 1993). Membrane fusions related to influenza HA have been studied in many systems, including fusions of virus, virosomes (reconstitution of viral envelopes), HA expressing cells with cultured cells, erythrocytes, and liposomes (with or without a specific receptor).

Major results or conclusions to date from above studies on HA related membrane fusions can be generally summarised (also see Siegel(1993)) as follows:

1. Fusion is initiated at low pH after exposure of the N-terminus of HA2 (fusion peptide). The complete dissociation of the top of HA trimer seems not necessary for HA induced membrane fusion (Stegmann et al., 1990).

2. Photolabeling experiments imply that N-terminus of HA2 bind to the lipid-water interface of the target membrane in advance of actual fusion. The N-termini appear to associate with the lipid-water interface more than with the interior hydrocarbon matrix of the target membrane (Harter et al., 1988; Harter et al., 1989; Stegmann et al., 1991; Brunner & Tsurudome, 1993).

3. By measuring fusion at low temperature (0°C), a delay (or lag phase) for the onset of fusion has been found (Morris et al., 1989; Sarkar et al., 1989; Stegmann et al.,
1991). It was found that the fusion peptides were inserted early in the lag phase (Stegmann et al., 1991). The lag is low-pH dependent, cumulative, and irreversible. More detailed descriptions of the lag phase are available in (Clague et al., 1993; Stegmann & Helenius, 1993). The lag phase preceding fusion may represent the time needed for bound trimers to assemble into a functional fusion complex (Stegmann et al., 1990, 1991; Spruce et al., 1989).

4. A concerted action of several HA trimers (which have to aggregate and interact in the membrane) is involved in the fusogenic activity (Morris et al., 1989; Ellens et al., 1990).

5. Influenza virus loses fusion activity very rapidly and irreversibly when incubated at low pH in the absence of target membranes (Sato et al., 1983a; Junankar & Cherry, 1986; Stegmann et al., 1987). It is suggested that dissociation of the tops of HA trimer is not only unnecessary for fusion, but probably renders HA incapable of mediating fusion (Stegmann & Helenius, 1993). The inactivation of influenza virus of subtype H3, after exposure to low pH at elevated temperatures (room temperature, 37°C) (White et al., 1982b; Stegmann et al., 1987), coincides with the opening of the tops and with an altered spike morphology (Doms et al., 1985; Ruigrok et al., 1984, 1984; Stegmann et al., 1987). For H2 subtype influenza virus, even after a 15-min incubation at pH 5.0 and 37°C, fusion activity is retained, while the top domains of HA trimer do not dissociate during this time. The inactivation is most likely caused by irreversible aggregation of viral HA, involving hydrophobic interactions between adjacent fusion peptides (Stegmann et al., 1987; Junankar & Cherry, 1986).

6. It is proposed that mobility of hemagglutinin is significant for its fusion activity. When pH is decreased from 7.3 to 5.2, the mobility observed at higher
temperature is required for the molecular rearrangements which accompany the fusion event. In the absence of an apposing target membrane, these rearrangements result in irreversible aggregation of hemagglutinin in the viral membrane, and hence loss of mobility and activity (Junankar & Cherry, 1986). Gutman et al. (1993) demonstrated that HA at the fusion-active state shows high lateral mobility at the surface of CV-1 cells, as required if the lateral motion of the fusion-promoting proteins indeed plays a role in the fusion mechanism. For X:31 HA, the lateral diffusion is inhibited only after the fusion reaction has occurred. They demonstrated a close correlation between loss of mobility and inactivation of fusogenic activity, in accord with the notion that lateral motion of the HA trimers is required for fusion. The need for lateral mobility of HA proteins may reflect the participation of several HA trimers in the active fusion complex, thereby requiring their relative motion to enable formation of the final fusion complex, and this view is further supported by Ramalho-Santos et al. (1993).

7. At low pH, BHA can form hydrophobic associations with lipid vesicles, or with nonionic detergent micelles and aggregates as protein-protein rosettes (Skehel et al., 1982). Treatment of these aggregates with thermolysin results in resolubilization of the protein rosettes, which requires removal of the first 23 residues of N-terminal peptide of HA2. These data indicate that the N-terminal region of HA2 is responsible for the hydrophobic character and aggregations of BHA at the pH of fusion (Daniels et al., 1983; Ruigrok et al., 1988). The formation of rosette-like aggregates in the viral membrane after acidification, as well as the tendency of acidified HA trimers to aggregate in detergent solution, have been reported by Doms and Helenius (1986).

Various models on HA induced membrane fusion have been proposed. While those models are common in the possible cooperativity of several HA trimers to form
a fusion pore (White, 1992), there are disagreements on how to determine the position of the fusion peptide of HA at fusogenic state. From the known neutral form structure of HA, the fusion peptide is about 35 Å away from the viral membrane and 100 Å away from the distal tips of HA1 globular head domains. How can the fusion peptides of HA trimers interact with target membrane or viral membrane and promote membrane fusion at low pH? What is the structure of fusion complex of HA trimers?

Stegmann et al. (1990) propose that the N-termini of HA2 inserts into both target and viral membranes to initiate the fusion process, and that the HA molecules must bend over to permit the insertion. Siegel (1993) modifies above model with a proposal that the inserted N-termini induce the lipids to form a stalk between the apposed bilayer. However, this raises questions about how the trimeric HA structure would bend substantially relative to the plane of the viral membrane. Wilschut and Baron (1993) modifies Stegmann's model such that the HA molecules are not bent, but propose that the propensity of the HA2 N-termini to insert into both target and viral membranes causing the tilting of the membranes, thus inducing a large bilayer curvature and fusion. Bentz et al. (1990) propose that, the HA molecules remain standing up and the exposed N-termini of HA2 initiate fusion by dehydrating the intermembrane space and inducing the lipid to "wet" the inner surface of the collar composed of aggregated HAs. This model is especially appealing for those fusion proteins that have no fusion peptide.

Chavela and Kim (1993) propose an interesting model that in the fusogenic state, the HA1 subunits are dissociated from the fibrous stem of HA. The fusion peptide is released from the protein interior, and the loop connecting the short α-helix and the long α-helix of HA2 "spring" into a helical conformation to form an extended coiled
coil that relocates the fusion peptide 100 Å towards the target membrane to promote membrane fusion. This model is quite appealing since the fusion peptide can interact with target membrane without the tilting of HA molecules. However, this model is based mainly on theoretical analysis and experiments with synthetic peptides. Obviously, more work is required to fully explain the action of HA molecules. Indeed, Ruigrok et al. (1986) demonstrated a thinning and a marked elongation of both HAs and BHAs at low pH.

One unanswered question is, while the N-terminal region of HA2 is responsible for the aggregations of BHA at low pH, no fusion model of HA so far has explained why or how, after the insertion of the fusion peptides into the target or viral membranes at low pH, HA trimers aggregate to form the fusion complex? This may be the crucial point to understand the mechanism of HA induced membrane fusion.

4.1.1.2 HIV virus

Human immunodeficiency virus (HIV) is an enveloped retrovirus in the lentivirus subfamily, which has a spherical shape about 100 nm in diameter. HIV virus was discovered to be the causative agent of acquired immunodeficiency syndrome (AIDS) in 1983 (Barré-Sinoussi et al., 1983; Gallo et al., 1983).

The viral spike protein precursor, gp 160, is an integral membrane protein of 856 amino acids. During or immediately after transport to the plasma membrane, gp 160 is cleaved into the two mature envelope glycoproteins, gp 120, a peripheral protein of 510 amino acids, and gp 41 comprising the membrane-anchored remaining 346 amino acids (Haffar et al., 1988). These two glycoproteins are held together by noncovalent interactions (Kowalski et al., 1987).

The cleavage of gp160 is required for the activation of HIV virus (McCune et
The N-terminus of gp41 consists of a conserved hydrophobic stretch of about 30 amino acids. Mutations within this domain which increase hydrophobicity tend to enhance syncytium formation (Bosch et al., 1989), while charged substitutions (Freed et al., 1990) or insertions of any kind can block syncytia (Kowalski et al., 1987; Bosch et al., 1989). It is generally considered that the amino terminus constitutes the fusion domain or "fusion peptide" of immunodeficiency viruses, by analogy to the N-terminal sequences of influenza HA2 protein and the membrane-anchoring subunits of the fusion proteins of other paramyxoviruses and orthomyxoviruses (Gallaher, 1987; Gonzalez-Scarano et al., 1987). The precise role of such "fusion peptides" in the fusion mechanism is being debated (Larsen et al., 1992; Bentz et al., 1990) and there is no direct evidence for this (Moore et al., 1993).

The existence of oligomeric gp120/41 complexes has been demonstrated (Pinter et al., 1989; Earl et al., 1990). The regions of gp41 that are responsible for oligomerization are unknown, but are likely to be within the extracellular portion of the molecule since secreted forms of gp160 truncated at the plasma membrane are aggregated (Berman et al., 1988, 1989; Earl et al., 1991). There is much less information concerning the oligomerization of gp120/41 than about influenza HA trimer.

The CD4 antigen acts as the cell surface receptor for HIV virus on lymphocytes, monocytes (Klatzmann et al., 1984; Dalgleish et al., 1984; McDougal et al., 1986), and other cells (Maddon et al., 1986; Gartner et al., 1986; Asjö et al., 1987). CD4 is a cell surface glycoprotein of 55 kDa. It is a member of the immunoglobulin superfamily. The binding between HIV virus and CD4 molecule is mediated by the surface glycoprotein gp 120 (Sattentau & Weiss, 1988). In addition to its use of CD4
as a productive receptor, HIV can also exploit other mechanisms to infect a cell (Takeda et al., 1988; Clapham et al., 1989; Harouse et al., 1989).

CD4 can induce conformational changes in gp120/41 after binding. The end result of the conformational change at 37°C is a dissociation of the gp120/41 complex, which leads to viral inactivation. At 4°C, there is no soluble CD4-induced removal of gp120 from HIV-1 infected cells (Hart et al., 1991) or from virions (Moore et al., 1990) at the time scale of release observed at 37°C. Soluble CD4-induced dissociation of gp120 from gp41 is also associated with the exposure of previously cryptic epitopes in the N-terminal ectodomain of gp41 (Hart et al., 1991; Sattentau & Moore, 1991), which contains a putative fusogenic sequence (fusion peptide). This exposure occurs even at 4°C, where there is no dissociation of the gp120/41 complex (Sattentau & Moore, 1991). It was speculated that a CD4-induced conformational change in the gp120/41 complex results in exposure of the fusogenic sequence at the N-terminus of gp41 and is an integral part of the fusion mechanism of HIV (Hart et al., 1991; Sattentau & Moore, 1991; Ellens & Larsen, 1993).

Whether the complete shedding of gp120 from gp41 is necessary for fusion is not known (Moore et al., 1993). It is also not known whether soluble and cellular CD4 behave equivalently, and whether only a small percentage of gp120 in a fusion complex may need to dissociate from gp41. It was assumed that at least in some regions of the contact zone between HIV virus and the attached cell, both gp120 and CD4 must be cleared from the intermembrane space, if only to allow the two membranes to approach one another physically (Moore et al., 1993).

HIV virus infection of CD4-bearing cells occurs by a pH-independent mechanism (Stein et al., 1987; McClure et al., 1988; Sinangil et al., 1988). HIV* (Ellens & Larsen, 1993)

* (Ellens & Larsen, 1993)
Virions can enter cells by two ways: direct fusion of the viral envelope with the cell membrane and receptor-mediated endocytosis via clathrin-coated pits and vesicles (Grewe et al., 1990). Endocytosis appears not to be an obligatory requirement of HIV virus infection in all cell types (Kielian & Jungerwirth, 1990).

Since the crystal structure of only first two domains of CD4 has been obtained, much research has concentrated on gp120/CD4 bindings with epitope mapping and mutagenesis. Compared with influenza HA induced fusion, there are much more unknowns in HIV virus induced fusion, especially regarding the interactions which lead to membrane fusion.

4.1.1.3 Viral fusion peptides

In view of the complexity of these systems and crucial role of fusion peptide in HA and gp41 proteins induced membrane fusion, synthetic (fusion) peptides corresponding to the sequence of these fusion peptide have been used to study the interactions between the fusion peptides and certain model membrane systems (liposomes or lipid monolayer). These model systems are relatively simple and direct methods (including spectroscopic methods) are available to study such interactions (Lear & DeGrado, 1987; Murata et al., 1987; Wharton et al., 1988; Lear & Rafalski, 1993).

Synthetic peptides corresponding to the HA2 N-terminal sequence of influenza virus hemagglutinin have been found to be able to induce fusion of sonicated vesicles of phosphatidylcholine (PC). The fusion could be in a pH dependent way which is similar to the parent virus (A/PR/8/34) (Murata et al., 1987), or in a way which is different from the parent virus (Lear & DeGrado, 1987; Wharton et al. 1988). The peptides H-16 and H-20, which are corresponding to the first 16 and 20 respectively, of B/Lee/40 strain of influenza HA2 were studied by Lear & DeGrado (1987). H-20
peptide, but not H-16 peptide, promotes fusion of sonicated 1-palmitoyl-2 oleyl phosphatidylcholine (POPC) vesicles, and H-20 peptide fuses the vesicles with a rate which is independent of pH between 5.0 and 7.0 whereas the parent protein is active only at pH < 6.0. The peptides corresponding to the sequence of the N-terminus of the HA2 subunit of influenza virus hemagglutinin (strain X31) could fuse cholesterol-free POPC liposomes at neutral as well as acid pH; however, POPC liposomes containing cholesterol could only be fused below pH 6 (Wharton et al., 1988).

It is proposed that the low pH trigger required for influenza viral infection is the conformational change of the HA which exposes the HA2 N-terminal peptide, rather than an obligatory requirement for the protonation of charged residues in the fusion process (Lear & DeGrado, 1987; Wharton et al., 1988). However, this proposition is contradictory to the results reported by Murata et al. (1987), who have found that the fusion can be rapidly switched on and off by adjusting the pH, to the acidic side and neutral, respectively. The peptide with an acetylated or succinylated N-termins also showed low pH-induced fusion activity but the pH range was shifted by 1 unit to the acidic side. The results indicate the protonation of the acidic residues in the segment is required for the activity. Despite the difference on pH dependence of the fusion induced by these synthetic peptides, these results all indicate that the HA2 N-terminal peptide of influenza virus hemagglutinin is directly involved in the fusion reaction (Murata et al., 1987; Lear & DeGrado, 1987; Wharton et al., 1988).

Peptides corresponding to the HA2 N-terminal segment of mutant influenza virus hemagglutinin were found to have fusion properties similar to the whole mutant hemagglutinin molecules (Wharton et al., 1988; Gething et al., 1986). Positive and negative charged amino acid residues have been introduced to replace certain residues
in the wild type synthetic fusion peptide to produce positive charged or negative charged peptides by Takahashi (1990), which could improve the solubility of these peptides.

Conformations of these peptides with or without lipid membranes have been studied with circular dichroism spectroscopy (CD) (Lear & DeGrado, 1987; Wharton et al., 1988; Takahashi, 1990; Burger et al., 1991; Rafalski et al., 1991; Epand et al., 1992). Lear and DeGrado (1987) showed that the H-20 peptide appears to bind lipid membranes in a helical conformation while H-16 probably binds in a more extended configurations, suggesting that helix formation is required for fusion activity. It is proposed that the fusion process involves formation of a membrane-binding helix which helps promote fusion by destabilizing the membrane (Lear & DeGrado, 1987). A correlation between the α-helical content of peptide and its fusogenicity was noted by Wharton et al. (1988), but this was not absolute. Takahashi (1990) concluded that α-helix formation was one of the necessary conditions to trigger a process of membrane fusion, since in his experiments, the peptides which are active to induce membrane fusion took an α-helical conformation in the presence of phospholipid bilayers, while a peptide which was unable to induce membrane fusion was in a β-structure. However, the wild type fusion peptide (strain A/PR/8/34) in his experiment took a β-structure predominant conformation.

Burger et al. (1991) and Rafalski et al. (1991) studied membrane binding and conformational properties of peptides which have the same sequences, and their results thus can be compared. Burger et al. (1991) showed a high content of α-helical structure for the fusion-active (wild type (WT) peptide, virus strain X31) and one of the fusion-defective analogues (G1E peptide, substitution of the wt peptide at the position 1 with
glutamic acid residue). However, Rafalski et al. (1991) showed that E1 (G1E) peptide has partial β-structure while the wt peptide became predominantly α-helical at acidic pH. So far, no detailed studies of the conformations of these peptides interacting with lipid membranes by Fourier transform infrared spectroscopy have been done.

The studies of the interaction of the peptides with lipid monolayer by Burger et al. (1991) showed that the fusion-active analog (wt peptide) has greater surface activity than two other fusion-defective analogues (G1E and ΔG1 peptides). When pure peptides were spread at the air/water interface, the fusion active analog (Wt peptide) showed a higher collapse pressure and a greater limiting molecular area (Burger et al., 1991). Rafalski et al. (1991) showed that the X31 "wild type" (WT) peptide inserts into POPC large unilamellar vesicle (LUV) membranes in a strictly pH-dependent manner, penetration being marginal at pH 7.4 and significant at pH 5.0. Bilayer insertion was indicated from a blue shift in the intrinsic Trp fluorescence of the peptide and the induction of calcein leakage from POPC LUV and correlated well with the ability of the peptides at pH 5.0 to penetrate into POPC monolayers at initial surface pressures higher than 30 mN/m. In contrast, at pH 5.0, the E1 (G1E) peptide was found to bind less tightly to vesicles and to cause much less and slower leakage of POPC LUV, and showed much less lipid vesicles induced blue shift of its Trp fluorescence, and did not insert into POPC monolayers at surface pressures beyond 28.5 mN/m (Rafalski et al., 1991). The above observations are consistent with the result that substitution of glutamic acid for the glycine residue at the amino-terminus of HA2 of influenza hemagglutinin which is expressed in simian cells abolished all fusion activity (Gething et al., 1986).

Burger et al. (1991) proposed that a simple relation between α-helical content
and fusogenicity does not seem to exist. Instead, the extent of penetration, a defined tertiary structure or orientation of the α-helical peptide may be essential for its membrane perturbing activity.

Rafalski et al. (1990) studied the interaction of peptides representing the N-terminal 23 residues of the gp41 protein of LAV_{1a} and LAV_{mal} strains of HIV virus. The peptides are surface-active and penetrate lipid monolayers composed of negatively charged (1-palmitoyl-2-oleyl-phosphatidylglycerol, POG) but not neutral (POPC) lipids. Similarly, the peptides induce lipid mixing and solute (6-carboxyfluorescein) leakage of POG, but not POPC sonicated vesicles. Martin et al. (1993) showed that a 16 residues peptide corresponding to the N-terminal sequence of gp41 protein of strain HXB2 HIV virus could only induce fusion of LUV vesicles which contain PE but not vesicles which only contain neutral lipid.

It is suggested that high concentrations of negatively charged lipids can artificially enhance the rate and extent of fusion by allowing non-physiologic fusion reactions to occur (Stegmann et al., 1989; White, 1990). Unsaturated PE could adopt hexagonal (H_{II}) phase, and lipid polymorphism is closely correlated with model membrane fusions (Cullis & Hope, 1988). Stegmann (1993) concluded that influenza hemagglutinin-mediated membrane fusion does not involve inverted phase lipid intermediates, since influenza virus fused efficiently with liposomes consisting either of saturated PC or PC/ganglioside mixtures, even when the liposomal membranes were in the gel phase and thus far from gel/H_{II} transitions. It will be interesting to know whether similar phenomenon can be found in HIV gp41/gp120 induced membrane fusion process. Before this question is answered, whether the introduction of POG or PE into LUV vesicles will enhance the rate and extent of fusion artificially should be
treated cautiously.

Sonicated small unilamellar vesicles (SUV) were suggested to be intrinsically unstable, and are highly prone to undergo fusion (Wilshut & Hoekstra, 1986; Wilshut, 1990), as this relieves the built-in packing imperfections of the lipids in their highly curved bilayer. These vesicles are unstable below the phase-transition temperature (Wilshut & Hoekstra, 1986), fuse under conditions in which larger liposomes only aggregate, and can fuse spontaneously both above and below their phase-transition temperatures (Lentz et al., 1987; Wilshut & Hoekstra, 1986). Stegmann et al. (1989) recommended that LUV and multilamellar vesicles (MLV) should be used for fusion studies, as their properties more closely approach those of natural membranes. Rafalski et al. (1991) proposed that the discordant reports (Murata et al., 1987; Lear & DeGrado, 1987; Wharton et al., 1988) concerning the pH dependence of the membrane-penetrating properties of various HA2-derived peptides resulted from using SUV vesicles to study fusion. However, it is unlikely to be true since Murata et al. (1987) also used SUV vesicles. The possible differences between them may be the different stock solutions they used to solubilise the peptides. Murata et al. (1987) used 5 mM formic acid-ammonia buffer, pH 7.8, 140 mM NaCl, or borate buffer, pH 9.2 (Murata, personal communication) as peptide stock solution, while Lear & DeGrado (1987) and Wharton et al. (1988) used DMSO. The negative charged side chains of acidic amino acid residues of the peptides may have different properties when the peptides are in DMSO but not in aqueous solutions.

It may be not a surprise that peptides representing the N-terminal 23 residues of the gp41 protein of LAV_{1a} and LAV_{mal} strains of HIV virus and wt peptide (Rafalski et al., 1990, 1991) failed to induce fusions of neutral phospholipid LUV vesicles.
Epand et al. (1992) have discussed the limitations of using peptide models to mimic viral fusion. Since many features of intact virus are not present in the simple synthetic peptide system, it is unlikely that a synthetic peptide could fully mimic viral fusion process. Either using SUV vesicles or introducing PE or negative charged lipid in LUV vesicles may all enhance the rate and extent of fusion. Whether using PE or negative charged lipid in LUV vesicles are biologically relevant remain uncertain. Compared with the induced lysis of liposomes or cells, the fusion of SUV vesicles induced by a fusion peptide may reflect more of its fusogenicity since to induce fusion of SUV vesicles the peptide needs to overcome hydration force of liposomal membranes to bring the membranes to be aggregated together, which is an essential step in a membrane fusion process. Many peptides or proteins, however, can induce lysis of liposomes or cells, but could not induce the fusion of them at all. Despite the limitations, studies of the model systems of synthetic fusion peptides can still provide useful direct information regarding the molecular mechanism of the interactions between fusion peptides and target membranes (Epand et al., 1992).

There are different ways to reconstitute a peptide with lipid membranes, depending on the solubility of the peptide in different solvents. A common way is to add the stock solution of the peptide to liposome suspension directly, and mixed well. In this way, some peptide molecules may interact with each other first before interacting with the lipid membrane (especially for hydrophobic peptides). Unless some separation procedures are used to separate those peptide molecules which interact with each other from those peptide molecules which interact with the lipid membrane, the conformation showed by the structural study of the sample will be the mixture of the two. Other methods may use detergent to co-solubilize the peptide and the lipid, then
the detergent is removed through dialysis. The ideal way to reconstitute a peptide with lipid is to find a solvent which can co-solubilize the peptide and the lipid, then the solvent can be evaporated, the dried films of the peptide-lipid mixture can then be hydrated to form a suspension consisting of lipid-peptide membrane fractions.

CD and infrared spectroscopy show that at low peptide:lipid ratios (~ 1:200), the peptides representing the N-terminal 23 residues of the gp41 protein of LAV\textsubscript{la} and LAV\textsubscript{mal} strains of HIV virus bind to POPG negatively charged vesicles as \( \alpha \)-helices. At higher peptide:lipid ratios (1:30), a \( \beta \) conformation is observed for the LAV\textsubscript{la} peptide, accompanied by a large increase in light scattering. The LAV\textsubscript{mal} peptide showed less \( \beta \)-structure and induce less light scattering. With POPC vesicles, only the \( \beta \) conformation and a peptide:lipid ratio-dependent increase in vesicle suspension light scattering were observed for both peptides. It is hypothesized that the inserted \( \alpha \)-helical form causes vesicle membrane disruption whereas the surface-bound \( \beta \) form induces aggregation (Rafalski \textit{et al.}, 1990).

A 16 residues peptide corresponding to the N-terminal sequence of gp41 protein of strain HXB2 HIV virus was shown by FTIR studies to adopt \( \sim 25\% \) \( \alpha \)-helix, 40-50\% \( \beta \)-sheet when interacting with LUV of PC/PE/SM/Chol (1/1/1/1.5 molar ratio) or LUV of PC/SM/Chol (2/1/1.5 molar ratio) (SM: sphingomyelin; Chol: cholesterol) (Martin \textit{et al.} 1993). Preliminary FTIR studies of the peptide representing the N-terminal 23 residues of the gp41 protein of LAV\textsubscript{la} strain of HIV virus in human red blood cell ghost lipids showed that when the peptide/lipid molar ratio = 1/200, the peptide had a dominant band which is a typical of disordered and \( \alpha \)-helical conformations, when the peptide/lipid molar ratio = 1/30, a further band appeared near 1634 cm\(^{-1}\), indicative of \( \beta \) strand in addition to disordered and \( \alpha \)-helical conformations (Gordon \textit{et al.}, 1992).
Wharton et al. (1988) reported that the peptides corresponding to the HA2 N-terminal segment of influenza virus hemagglutinin aggregate in solution. Similar aggregations of HIV gp41 N-terminal peptides in PBS solutions were also reported by Slepushkin et al. (1992). Wharton et al. (1988) suggested that this aggregation may enable the peptides to interact simultaneously with more than one liposome. Slepushkin et al. (1992) proposed that there are two stages in a process of the interaction between fusion peptide and lipid membranes: 1) membrane penetration by the peptides, 2) lipid intermixing. It was suggested that the two stages have different requirements for the peptide structures: The ability of peptides to penetrate the lipid bilayers correlates with their capacity to form α-helix structure in nonpolar media, and the lipid intermixing induced by the peptides correlates with their potential to form long filaments in aqueous solution, which also seems to be necessary for generation of fusion complex from glycoprotein spikes inserted in viral membrane (Slepushkin et al., 1992).

The occurrence of HIV N-terminal gp41 peptide multimers when the peptide is interacting with erythrocyte ghosts were reported by Gordon et al. (1992), and was suggested to be correlated with the coexistence of α-helix and β-strand structures of the peptide in red cell ghost lipid at the peptide/lipid molar ratio = 1/30. Gordon et al. (1992) speculated that aggregates of the N-terminal gp41 peptide in membranes may be due to the formation of multimers of α-helical peptides or β-strand peptides, or both. When the HIV N-terminal gp41 peptide interact with the membrane of intact human red cells at low peptide/lipid ratio, the peptide exists as a monomeric, α-helical structure, which does not cause lysis. When the peptide interact with human red cell membranes at higher peptide/lipid ratio, however, the peptides reside in red cell membranes and form multimeric aggregates containing both α-helical and β-strand structures, which
correlates well with the onset of haemolysis (Gordon et al., 1992). It was proposed that the cooperation of at least 3-4 influenza HA2 N-terminal peptide molecules is required to lower the energy barrier for fusion from the third to fourth order dependence of the fusion rate on the bound peptide concentration (Lear and DeGrado, 1987).

Theoretical analyses on the mode of insertion of the N-terminal fusion peptides of various virus predicted that the fusion peptides oriented obliquely with respect to the lipid/water interface (Brasseur et al., 1988, 1990; Horth et al., 1991) when the peptides were assumed to insert into lipid membranes as an α-helical structure. This rather unusual orientation is envisaged as prerequisite to membrane destabilization and fusogenic activity (Brasseur et al., 1990).

FTIR studies showed that the peptides corresponding to the gp32 N-terminus of SIVmac (simian immunodeficiency virus) adopt a secondary structure with a mixture of α-helix and β-sheet when it is interacting with POPC SUV vesicles. The α-helix was shown to be neither parallel nor perpendicular to lipid acyl chain but adopt an intermediate orientation in the lipid bilayer. The β-sheet was showed to be parallel to the lipid bilayer (Martin et al., 1991). The α-helix in the 16 residues peptide corresponding to the N-terminal sequence of gp41 protein of strain HXB2 HIV virus was showed to insert into the lipid bilayer with the helix axis orientated parallel to the lipid acyl chains when the peptide is interacting with LUV vesicles of PC/PE/SM/Chol (1/1/1/1.5 molar ratio), or to be parallel to the lipid/water interface when it is interacting with LUV vesicles of PC/SM/Chol (2/1/1.5 molar ratio), no significant orientation was revealed for the β-sheet structure (Martin et al. 1993). Whether any specific orientation of the fusion peptides in lipid membrane is prerequisite and crucial to its membrane destabilization and fusogenic activity, and whether the predictions made by theoretical
analyses on the orientation of the inserted fusion peptide in lipid membranes are valid still remain an open question.

In this chapter, the secondary structures and orientations of the fusion peptides corresponding to the N-terminus of HA2 protein of strains A/PR/8/34 and X31 influenza virus (HA and WT (wild type) peptides, respectively), a peptide with a substitution of glutamic acid for the glycine residue at the N-terminal of the wt peptide (G1E peptide), and the fusion peptide corresponding to the N-terminus of gp41 protein of ARV2 strain HIV-1 virus (HIV1 peptide) were investigated in egg phosphatidylcholine bilayers with polarised, attenuated total reflection (ATR), Fourier transform infrared spectroscopy (FT-IR). Both dried and hydrated samples were studied. The sequences of these peptides are shown in Figure 4.1.

Infrared spectroscopy is a vibrational spectroscopy which can be used to study the conformation of lipid molecules and membrane proteins in biomembranes simultaneously. The basic mechanism of FTIR spectroscopy and its application in studying the structure and function of biomembranes are thus introduced in Appendix I.

The results showed that the orientation of the α-helix in the influenza and HIV N-terminal synthetic fusion peptides was not completely perpendicular to the lipid membrane bilayer and while α-helix in the fusion peptides may play an important role in membrane fusion process, a possible role for β-sheet structures cannot be excluded. The functions of fusion peptides may be determined by multiple factors such as secondary structures, orientations, hydrophobicity, membrane insertion etc., but not solely by the content of α-helical structure. It seems that both the insertion of the fusion peptide into the target membrane, and the subsequent aggregation of hemagglutinin
trimers in intact influenza virus, are necessary for virus induced membrane fusion. It is quite possible that fusion peptides bind and insert into lipid membranes through their α-helices and are aggregated through their β-sheet structures.

4.2 Materials and Methods

4.2.1 Materials.

Egg Phosphatidylcholine (egg PC), Hepes, Deuterium oxide (D₂O) (99.9 atom % D), Deuterium chloride (DCl) (20 % solution in D₂O) were from Sigma Chemical Co. Dimethyl sulfoxide (DMSO) and 1,1,1,3,3,3-Hexafluoro-propan-2-ol (HFPO) were from BDH. Deionized distilled water was used throughout. N-(7-nitro-2,1,3-benzoxadiazol-4-yl)-dioleoylphosphatidylethanolamine (NBD-DOPE) and N-(lissamine Rhodamine B sulfonyl)-dioleoylphosphatidylethanolamine (rhodamine-PE) were from Avanti Polar Lipids (Birmingham, AL).

4.2.2 Peptides.

Figure 4.1 shows the sequences of the peptides. The HA peptide was kind gift from Dr. Murata of Kyoto University, and are >90% pure (Murata et al., 1987). The WT peptide and the G1E peptide were kind gifts from Dr. Wharton of National Institute for Medical Research, Mill Hill, London, and are >90% pure (Wharton et al., 1988).

HA       GLFGAIAGFIEGGWTGMDG
WT       GLFGAIAGFIENGWGMDG
G1E      ELFGAIAGFIENGWGMDG
HIV1     AVGIVGAMFLGFLGAAGSTMGAVALTLTVQA

Figure 4.1 Amino acid sequences of the peptides which were investigated in this chapter. Charged amino acid residues are underlined.

The HIV1 peptide was kind gift from Dr. Bansal of King’s College, London and
was synthesised on amilligen 9050 peptide synthesizer. HPLC characterization of the peptides was not possible because the peptide was very insoluble (not soluble in 0.1% trifluoroacetic acid in acetonitrile). Thus the peptide was used without further purification. The peptide was characterized by amino acid analysis, a 30 hour and a 24 hour hydrolysis was carried out (Table 4.1):

<table>
<thead>
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<th>24 hr</th>
<th>30 hr</th>
<th>Required</th>
</tr>
</thead>
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<td>3</td>
</tr>
<tr>
<td>Ser</td>
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<td>2</td>
</tr>
<tr>
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<td>1.11</td>
<td>1</td>
</tr>
<tr>
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<td>5.35</td>
<td>6</td>
</tr>
<tr>
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<td>6.00</td>
<td>6</td>
</tr>
<tr>
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<td>3.25</td>
<td>4</td>
</tr>
<tr>
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<td>0.62</td>
<td>1</td>
</tr>
<tr>
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<td>3.88</td>
<td>4</td>
</tr>
<tr>
<td>Phe</td>
<td>2.12</td>
<td>1.73</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 4.1 Amino acid analysis of HIV1 peptide after a 30 hr and a 24 hr hydrolysis.

4.2.3 Preparation of Liposomes.

For egg PC liposome containing 1 mol% each of NBD-DOPE and Rhodamine-DOPE, NBD-DOPE stock solution (1mg/1.5ml in methanol) and Rhodamine-DOPE stock solution (1mg/1.5ml in methanol) were mixed with egg PC in chloroform/methanol (v/v, 2/1), dried by nitrogen (N₂) current to form thin film, and kept under N₂ current for at least one hour. The lipid film was hydrated by water for HIV1 peptide sample, and 5 mM Hepes buffer at pH 7.4 for HA, WT, G1E peptide samples. The lipid concentration was 1 mg/ml. The suspensions were vortexed and sonicated in a water bath sonicator for at least 15 min + 15 min until the suspension was clear. The bottle containing the suspension was filled with N₂ and resealed before
the sonication. Egg PC liposome containing 0.1 mol % each of NBD-DOPE and Rhodamine-DOPE was prepared as above, except different amounts of NBD-DOPE and Rhodamine-DOPE were used. Egg PC liposome was prepared in a similar way but no probes were used.

4.2.4 Fluorescence Measurements.

Fluorescence spectra were measured with a Perkin-Elmer MPF-44B Fluorescence Spectrophotometer. A stirring apparatus located beneath the cuvette holder enabled constant mixing of the medium. The NBD fluorescence was measured at an excitation wavelength of 455 nm and an emission wavelength of 530 nm with both excitation and emission slits at 7 nm. The light scattering was measured with both excitation and emission wavelengths at 400 cm⁻¹, and with both excitation and emission slits at 2 nm.

Egg PC liposomes containing 1 % mol NBD-DOPE/Rhodamine-DOPE were mixed with unlabelled egg PC liposomes with a ratio of 1/9, water was added to the sample for HIV1 peptide, and 10 mM Hepes buffer at pH 7.4 was added for samples for HA, WT, G1E peptides. The final lipid concentration was 350 μM. The NBD fluorescence and light scattering of all samples were measured before and after the peptides were added. The HIV1 peptide was added from its DMSO stock solution and mixed well with the liposome suspensions in water, in the HIV1 peptide/egg PC molar ratio of 1/22. HA, WT, G1E peptides were added from their 10 mM borate pH 9.2 stock solutions and mixed well with the liposome suspensions in 10 mM Hepes buffer at pH 7.4, with the peptide/egg PC molar ratios also being 1/22. After the NBD fluorescence and light scattering of HA, WT, G1E peptide samples at pH 7.4 were measured, the pH of the suspensions were then adjusted to pH 5 by adding 1 M HCl and the NBD
fluorescence and light scattering of these samples at pH 5 were measured. The residual fluorescence of the sample before addition of peptides was set as 0% of maximum fluorescence ($F_0$). The fluorescence of 350 μM egg PC liposomes labelled with 0.1 % mol NBD DOPE/Rh-DOPE was taken as 100% maximum fluorescence ($F_{max}$). The lipid mixing (%) can be calculated as \( \{ \frac{F - F_0}{F_{max} - F_0} \} \times 100\% \).

4.2.5 Sample Preparation For FT-IR Measurements.

Peptides were solubilized in HFPO solvent as stock solution. 2 mg egg PC in chloroform was dried by N$_2$ current to form dry film. Peptides in stock solutions were mixed with the egg PC film and HFPO was used to co-solubilise the peptide and egg PC (Rafalski et al., 1991). The molar ratio of the peptide to egg PC was \( \sim 1/60 \) for WT, HA, and G1E peptides samples, and \( \sim 1/200 \) or 1/250 for HIV1 peptide sample. More chloroform was then added and the mixture was dried by N$_2$ current and kept under N$_2$ current for at least one hour. 1.6 ml H$_2$O was added to hydrate the HIV-1 peptide/egg PC sample. 1.6 ml 3 mM Hepes pH7.4 buffer or 1.6 ml H$_2$O containing dilute HCl at pH5 were added to hydrate the HA, WT, G1E peptide/egg PC samples. The suspensions were vortexed and stored under N$_2$.

4.2.6 FT-IR Measurements.

Infrared spectra were obtained with a Mattson Galaxy 4020 Fourier transform spectrometer. An ATR unit from Perkin-Elmer and a parallelogram-shaped germanium ATR crystal (50 x 20 x 2 mm, face angle 45°) were used for polarized ATR measurements. A grid polarizer (Spectra-TECH Inc.) was placed in front of the ATR unit. The measurements were undertaken at room temperatures and the spectrometer was continuously purged with dried air.

80 μl of sample (2 mg lipid/1.6 ml) was placed on a germinium crystal in a
desiccator, then the water was removed by a vacuum pump. The dried sample films had a uniform thickness and an area of $\sim 3.2 \text{ cm}^2$. The thickness of the films was estimated as $\sim 0.24 \mu\text{m}$.

Both ATR FT-IR and polarized FT-IR measurements were undertaken. For ATR measurements, no polarizer was used, background spectra were recorded with no ATR unit. For polarized ATR measurements, a polarizer was used and was set at 90° and 0° for parallel and perpendicular polarized infrared radiation respectively. Background spectra were recorded without ATR unit and polarizer. Crystal spectra were recorded with blank germanium crystal in the ATR unit with or without polarizer. Water vapour spectra were collected similarly to crystal spectra but, before the measurement was started, the instrument chamber was open for certain time to let water vapour to enter. 3000 - 4500 interferograms were typically collected at single beam mode, co-added, and apodized with a triangular function, and Fourier transformed to give 4 cm$^{-1}$ resolution.

Sample absorbance spectra were obtained from the ratio of the sample single beam spectra to background spectra. Crystal absorbance spectra were subtracted from the sample absorbance spectra to produce sample difference spectra, then water vapour spectra were subtracted from the difference spectra. Second derivative spectra were obtained with a 19-point Savisky-Golay smooth function and a Mattson second derivative routine program. Dichroic ratios of infrared bands were determined using method described in Appendix I.

To hydrate the sample film, a metal cell was mounted on the sample-covered side of the crystal, and different amounts of D$_2$O were added through the two inlets of the cell to ensure the proper hydration of the sample (according to the hydrophobicity and buffer concentrations of different samples. D$_2$O containing dilute deuterium
chloride at pH 5 was used to hydrate the samples at pH 5. D₂O was used for the hydration of the samples at pH 7.4 and HIV-1 peptide/egg PC sample.

4.3 Results

4.3.1 Liposome fusion induced by peptides

Lipid-mixing assays were used to monitor liposome fusion induced by fusion peptides. Figure 4.2 shows that lipid mixing occurred immediately after the addition of HIV-1 fusion peptides in DMSO stock solution to egg PC sonicated vesicles suspensions (1% mol NBD/Rhodamine egg PC vesicles : egg PC vesicles = 1 : 9). The lipid mixing reached a plateau within 30 min, and at 90 min the lipid mixing induced by HIV-1 peptide was (50 %). An immediate increase of light-scattering at 400nm was observed after egg PC sonicated vesicles were mixed with HIV-1 stock solution (not shown).

![Lipid Mixing Induced by HIV-1 Fusion Peptide](image)

**Figure 4.2** Lipid-mixing of sonicated egg PC vesicles by HIV-1 peptides. (○) HIV1 peptide; (●) DMSO control.
The lipid-mixing induced by WT, G1E and HA peptides was also checked. Lipid-mixing induced by HA peptide was in good agreement with the published results (Murata et al., 1987) with strict pH-dependence. There was no increase of NBD fluorescence and light scattering induced by WT peptide at pH 7.4, so WT peptide could not induce fusion of sonicated egg PC vesicles at pH 7.4. Lipid mixing and increase of light scattering of egg PC sonicated vesicles induced by WT peptide were observed at pH 5. No increase of NBD fluorescence and light scattering induced by G1E peptide at pH 7.4 was observed. There was only minor increase of NBD fluorescence induced by G1E peptide at pH 5. However, no increase of light scattering of the egg PC sonicated vesicles was observed (not shown), so G1E peptide could not induce fusion of egg PC sonicated vesicles at both pH 7.4 and pH 5. The pH dependence of the fusogenicities of WT and G1E peptides observed was thus different from the results reported by Wharton et al. (1988), possibly because different solvent systems were used to solubilise the peptides. The conformational properties of WT and G1E peptides in lipid membranes at pH 7.4 and pH 5 were therefore studied in egg PC lipid membranes, instead of in egg PC + cholesterol membranes.

4.3.2 ATR FT-IR spectroscopy.

For each sample studied both absorbance and second derivative spectra are shown. An absorbance unit (a.u.) is shown in each absorbance spectrum. The position (in wavenumber, cm\(^{-1}\)) of major bands is labelled in both absorbance and second derivative spectra; positions obtained from second derivative analysis are usually more accurate. The 1800-1900 cm\(^{-1}\) region of a second derivative spectrum shows the noise level of the spectrum. Only when the intensity of a band \( \geq 2 \times \) noise level, the band can be considered as signal rather than noise.
The bands arising from the peptides studied have been assigned according to Jackson et al. (1989). The band at ~ 1660 in dry samples, which was shifted to ~1650 cm\(^{-1}\) after hydrated by D\(_2\)O (due to deuterium/hydrogen exchange), is assigned to \(\alpha\)-helix. The band at ~1626 cm\(^{-1}\) is assigned to \(\beta\)-sheet. The band at ~1691 cm\(^{-1}\) is assigned to \(\beta\)-sheet or turns structure. The band at ~1543 cm\(^{-1}\) is assigned to amide II band. Individual assignments were made for the absorbance bands from each peptide studied.

When trifluoroacetic acid (TFA) was added to a peptide-lipid suspension, after the sample was dried on a Ge crystal, an extra sharp band at 1688 cm\(^{-1}\) in the absorbance spectrum was seen (not shown). After the sample was hydrated by D\(_2\)O or H\(_2\)O, the band at 1688 cm\(^{-1}\) was shifted to ~1673 cm\(^{-1}\). A synthetic peptide may contain traces of TFA which may contribute to an band at ~1673 cm\(^{-1}\) (Fabian et al., 1993). Since this band overlaps with the band assigned to turns structure, the assignment of bands at ~1673 cm\(^{-1}\) should be made with care. Efforts were made to get rid of contaminating TFA in HIV1 peptide. The HIV1 peptide was washed with 1 M NaHCO\(_3\) solution more than 8 times, then washed with deionized water more than 10 times (the HIV1 peptide is very hydrophobic and insoluble in water), and the peptide suspension in water was then lyophilised.

The carbonyl band of egg PC in all dry samples had similar position at ~ 1739 cm\(^{-1}\). After the samples were hydrated, the carbonyl band of egg PC was shifted to lower frequency ~1732 cm\(^{-1}\), and second derivative spectra revealed the existence of two bands at ~1742 cm\(^{-1}\) and at ~1729 cm\(^{-1}\). This is due to the hydrogen bonding of water molecules to the C=O groups (Blume et al., 1988; Hübner & Mantsch, 1991), which results in the appearance of the low frequency component band at ~1729 cm\(^{-1}\).
The band at $\sim 1742$ cm$^{-1}$ is due to the free carbonyl group (Blume et al., 1988). Thus these two bands arise from C=O groups of egg PC which are hydrated to different extents, and the band at $\sim 1729$ cm$^{-1}$ is the more hydrated. The relative ratio of these two bands can be used to estimate the hydration state of carbonyl group of egg PC molecules.

4.3.2.1 *HA peptide in lipid membranes*

HA peptide was reconstituted in egg PC lipid membranes at both pH 7.4 and pH 5. The suspension formed at pH 5 was more cloudy than at pH 7.4, indicating the WT peptide in the membranes may induce aggregation or fusion at pH 5, which is consistent with the pH dependence of fusogenicity of the HA peptide observed in the lipid-mixing assay. However, the absorbance and second derivative spectra of the dry films of HA peptide in egg PC lipid membranes at pH 7.4 (Figure 4.3 a & b) and at pH 5 (Figure 4.5 a & b) show a similar secondary structure of the peptide at both pH, which is predominantly $\alpha$-helical (a major band at $\sim 1660$ cm$^{-1}$) with minor $\beta$-sheet structure (bands at $\sim 1626$ cm$^{-1}$ and $\sim 1691$ cm$^{-1}$). The minor $\beta$-sheet might be due to the aggregation of a small amount of the peptide during the sample preparation.

The dried sample films of HA peptide with egg PC at pH 7.4 and pH 5 were hydrated with 25 $\mu$l D$_2$O and 94 $\mu$l D$_2$O+DCl (pD 5), respectively. The absorption and second derivative spectra of the sample films of HA peptide in egg PC lipid membranes at pH 7.4 and pH 5 after hydrated for 5 hours are shown in Figure 4.4 (a & b) and Figure 4.6 (a & b), respectively. The band at $\sim 1660$ cm$^{-1}$ in the dry sample was shifted to $\sim 1651$ cm$^{-1}$ due to deuterium/hydrogen (D/H) exchange after hydration. The band at $\sim 1691$ cm$^{-1}$ in the dried samples at both pH was initially shifted to $\sim 1675$ cm$^{-1}$ after hydration. Then the intensity of the band or shoulder at $\sim 1688$ cm$^{-1}$ of the
Figure 4.3 The absorption (a) and second derivative (b) spectra of dry HA peptide-egg PC membrane film at pH 7.4 (molar ratio: HA peptide/egg PC ≈ 1/60).
Figure 4.4 The absorption (a) and second derivative (b) spectra of HA peptide-egg PC membrane film at pH 7.4 hydrated by 25 μl D₂O for 5 hours (molar ratio: HA peptide/egg PC ≈ 1/60).
Figure 4.5 The absorption (a) and second derivative (b) spectra of dry HA peptide-egg PC membrane film at pH 5 (molar ratio: HA peptide/egg PC \( \approx 1/60 \)).
Figure 4.6 The absorption (a) and second derivative (b) spectra of HA peptide-egg PC membrane film at pH 5 hydrated by 94 µl D_2O+DCl (pD 5) for 5 hours (molar ratio: HA peptide/egg PC ≈ 1/60).
hydrated sample (at both pH) increased in parallel with the increase of the intensity of the band at 1626 cm\(^{-1}\) when the hydration time was longer. The formation of \(\beta\)-sheet structure after hydration at both pH were shown by the appearance of bands at \(\sim 1626\) cm\(^{-1}\) (at both pH) and \(\sim 1688\) cm\(^{-1}\) (at pH 7.4). When the sample films are rehydrated, tight contact between lipid membrane layers may enable peptide molecules which are anchored to the membranes to interact with each other and form aggregates through the formation of inter-molecular \(\beta\)-sheet.

Although more D\(_2\)O+DCI (94 \(\mu\)l) was used to hydrate the sample at pH 5, the sample at pH 5 was still less hydrated than the sample at pH 7.4 (hydrated by 25 \(\mu\)l D\(_2\)O), as was shown by the lower ratio of two bands (at \(\sim 1728\) cm\(^{-1}\) / \(\sim 1742\) cm\(^{-1}\)) from egg PC carbonyl group in the sample of pH 5 (shown in second derivative spectra Fig. 4.6b and Fig. 4.4b, respectively). The increase of the hydrophobicity of the peptide-lipid membranes is due to protonation of the acidic residues in the HA peptide at pH 5 (Murata et al., 1987). This observation indicates that HA peptide molecules penetrate into the lipid membranes at least to the position of egg PC carbonyl group.

The minor bands at 1672 cm\(^{-1}\) of the hydrated sample at pH 7.4 (Fig. 4.4b) and the band at 1676 cm\(^{-1}\) of the hydrated sample at pH 5 (Fig. 4.6b) are assigned to turns structure since there is no indication of contamination of TFA in this peptide. The amide II band in the hydrated samples at both pH disappeared due to the deuterium/hydrogen (D/H) exchange, which indicates that in both cases the HA peptide was not fully inserted into the lipid membranes. The broad bands at \(\sim 1591\) cm\(^{-1}\) and \(\sim 1562\) cm\(^{-1}\) of the hydrated sample at pH 7.4 were due to side-chain absorptions of aspartic acid and glutamic acid residues respectively (Fabian et al., 1992). The absence of the these two side-chain absorption bands at the hydrated sample at pH 5 may reflect
the different pH of the two samples.

4.3.2.2 *Wild type peptide in lipid membranes*

Similarly to the HA peptide, the wild type (WT) peptide-egg PC suspension at pH 5 was more cloudy than at pH 7.4, which is consistent with the lipid-mixing assay for this peptide. The absorbance spectra of the dry films of WT peptide in egg PC lipid membranes at pH 7.4 and pH 5 are shown in Fig. 4.7a and Fig. 4.9a, respectively. The difference between the two spectra is that there is a stronger shoulder at 1685 cm\(^{-1}\) in the spectrum of WT peptide at pH 5. Second derivative spectra of these two samples are shown in Fig. 4.7b (pH 7.4) and Fig. 4.9b, respectively. WT peptide in egg PC membranes at pH 7.4 is predominantly α-helical (a major band at 1657 cm\(^{-1}\)) with a minor band at 1688 cm\(^{-1}\). WT peptide in egg PC membranes at pH 5 is also predominantly α-helical (a major band at 1657 cm\(^{-1}\)) with a relatively strong band at 1689 cm\(^{-1}\). The band at \(~1688\) cm\(^{-1}\) could be assigned either to turns structure or to contaminating TFA. If this band were due to TFA, then the difference between these two samples may be not relevant to the difference of their fusogenicities, but it is not known why the band at 1689 cm\(^{-1}\) of the sample at pH 5 was much stronger than the band at 1688 cm\(^{-1}\) of the sample at pH 7.4, since same peptide was used in both samples.

The dry sample film of WT peptide with egg PC at pH 7.4 was hydrated with 5 µl D\(_2\)O. Figure 4.8a shows the absorption spectrum of WT peptide in egg PC lipid membranes at pH 7.4 after being hydrated for 4 hours. The amide I band maximum was shifted to 1651 cm\(^{-1}\). A new band at 1626 cm\(^{-1}\) appeared, which is assigned to β-sheet. The amide II band disappeared after the D/H exchange, indicating the peptide was not deeply inserted in the lipid membranes. The broad band between 1550 cm\(^{-1}\)
and 1600 cm\(^{-1}\) was due to the side-chain absorptions of aspartic acid and glutamic acid residues (Fabian et al., 1992). The second derivative spectrum shows that the band at 1688 cm\(^{-1}\) of the dry film was shifted to 1675 cm\(^{-1}\) with a shoulder at\(\sim\)1690 cm\(^{-1}\). The band at 1661 cm\(^{-1}\) of the dry sample film was shifted to 1652 cm\(^{-1}\) after hydration with D\(_2\)O, and is confirmed to be \(\alpha\)-helix.

The dry sample film of WT peptide with egg PC at pH 5 was hydrated with 11 \(\mu\)l D\(_2\)O+DCI (pD 5). Figure 4.10a shows the absorption spectrum of WT peptide in egg PC lipid membranes at pH 5 after being hydrated for 4 hours. The amide I band maximum was shifted to 1653 cm\(^{-1}\) and no formation of \(\beta\)-sheet was observed. The amide II band did not completely disappear after the D/H exchange for 4 hours, probably because the sample is relatively hydrophobic. No side-chain absorptions of aspartic acid and glutamic acid residues were observed at pH 5. The second derivative spectrum shows that the band at 1688 cm\(^{-1}\) of the dry film was shifted to 1675 cm\(^{-1}\) with a shoulder at\(\sim\)1690 cm\(^{-1}\) (Fig. 4.10b), which is similar to the hydrated sample at pH 7.4. The band at 1657 cm\(^{-1}\) of the dry sample film (pH 5) was shifted to 1653 cm\(^{-1}\) after hydration with D\(_2\)O, and is confirmed to be \(\alpha\)-helix. A minor shoulder at 1639 cm\(^{-1}\) might be due to turns or \(\beta\)-sheet structure (Jackson et al., 1989). The egg PC carbonyl group C=O was less hydrated than in the sample at pH 7.4.

In a separate experiment, the dry sample film of WT peptide with egg PC at pH 5 was hydrated with 92 \(\mu\)l D\(_2\)O+DCI (pD 5). Figure 4.11a shows the absorption spectrum of WT peptide in egg PC lipid membranes at pH 5 after hydration for 6 hours. The amide II band disappeared after D/H exchange for 6 hours indicating that, although the sample is relatively hydrophobic, the peptide was not deeply inserted in the lipid membrane. The second derivative spectrum shows the appearance of a band at
Figure 4.7 The absorption (a) and second derivative (b) spectra of dry WT peptide-egg PC membrane film at pH 7.4 (molar ratio: WT peptide/egg PC $\approx 1/60$).
Figure 4.8 The absorption (a) and second derivative (b) spectra of WT peptide-egg PC membrane film at pH 7.4 hydrated by 5 μl D₂O for 4 hours (molar ratio: WT peptide/egg PC ≈ 1/60).
Figure 4.9 The absorption (a) and second derivative (b) spectra of dry WT peptide-egg PC membrane film at pH 5 (molar ratio: WT peptide/egg PC $\approx 1/60$).
Figure 4.10 The absorption (a) and second derivative (b) spectra of WT peptide-egg PC membrane film at pH 5 hydrated by 11 µl D$_2$O+DCl (pD 5) for 4 hours (molar ratio: WT peptide/egg PC \( \approx 1/60 \)).
Figure 4.11 The absorption (a) and second derivative (b) spectra of WT peptide-egg PC membrane film at pH 5 hydrated by 92 μl D_2O+DCl (pD 5) for 6 hours (molar ratio: WT peptide/egg PC ≈ 1/60).
1687 cm\(^{-1}\), which is assigned to \(\beta\)-sheet. The intensity of this band increased in parallel
to the increase of the \(\beta\)-sheet band at 1626 cm\(^{-1}\) and to the decrease of the band
at \(\sim 1675\) cm\(^{-1}\). This indicates that the band at \(\sim 1675\) cm\(^{-1}\) in the hydrated sample (at
both pH) and the band at \(\sim 1688\) cm\(^{-1}\) in the dry sample film (at both pH) is at least
partially due to turns structure since it is unlikely that a band at \(\sim 1673\) cm\(^{-1}\) due to
TFA will shift back to 1688 cm\(^{-1}\) when it is still hydrated. It is interesting to find that
the egg PC carbonyl group C=O of the sample at pH 5 which was hydrated by 92 \(\mu\)l
D\(_2\)O+DCI (pD 5) (Fig. 4.11b) was less hydrated than in the sample at pH 5 which was
hydrated by 11 \(\mu\)l D\(_2\)O+DCI (pD 5) (Fig. 4.10b). This is probably due to the formation
of \(\beta\)-sheet in the former sample which made the sample more hydrophobic.

4.3.2.3 \(\textit{G1E peptide in lipid membranes}\)

The suspension of G1E peptide-egg PC at pH 5 was more cloudy than at pH
7.4, similar to WT peptide, but this was not consistent with the inability of the G1E
peptide to induce fusion. This may be due to when G1E peptide was reconstituted in
the lipid membranes in current method, the peptide could insert into the lipid
membranes in certain extent, but while the G1E peptide was added from its stock
solution to a liposome suspension, the negative charged glutamic acid residue at the N-
terminal of this peptide may prevent it insert into the lipid membranes.

The absorption and second derivative spectra of the dry films of G1E peptide
in egg PC lipid membranes at pH 7.4 and at pH 5 are shown in Figure 4.12 (a & b)
and Figure 4.14 (a & b). It appears that G1E peptide in the dry lipid films (at both pH)
adopted very similar secondary structure to the WT peptide (Fig. 4.7 (a & b) and Fig.
4.9 (a & b). This is consistent with the observations of CD spectroscopy by Burger \textit{et
The dry sample film of G1E peptide with egg PC at pH 7.4 was hydrated with 4.4 μl D2O. Figure 4.13a and 4.13b show the absorption and second derivative spectra of G1E peptide in egg PC lipid membranes at pH 7.4 after hydration for 5 hours. The secondary structure of G1E peptide in the hydrated lipid membranes at pH 7.4 is very similar to the secondary structure of WT peptide in the hydrated lipid membranes at pH 7.4 (Fig 4.8a and 4.8b). The dry film of G1E peptide with egg PC at pH 5 was hydrated with 94 μl D2O+DCI. The absorption and second derivative spectra of G1E peptide in egg PC lipid membranes at pH 5 after hydration for 3 hours are shown in Figure 4.15a and 4.15b. The strong sharp band at 1624 cm⁻¹ was due to aggregated β-sheet. The weak band at 1672 cm⁻¹ may indicate that the possible contamination of TFA was not as serious as that shown in the dry film spectrum (Fig. 4.14b). Alternatively, the band at ~1672 cm⁻¹ in the hydrated sample film at pH 7.4 (Fig. 4.13b) and the band at 1687 cm⁻¹ in the dry sample film at pH 5 (Fig. 4.14b) were partially due to turns structure, which is consistent with the structure of HA and WT peptides. The carbonyl group of egg PC in the hydrated sample film was less hydrated at pH 5 than at pH 7.4, which was similar to HA and WT peptide.

It appears that the formation of β-sheet in hydrated G1E peptide-egg PC membranes was quicker and more extensive at pH 5. It seems that although G1E peptide could be reconstituted in the lipid membranes using the procedure employed here, the charged residue at the N-terminal of G1E peptide may prevent the peptide from inserting deeply into the membranes. Consequently, it was easier for molecules of G1E peptide to diffuse on the surface of the lipid membrane to interact with one another to form aggregates through the formation of inter-molecular β-sheet.
Figure 4.12 The absorption (a) and second derivative (b) spectra of dry G1E peptide-egg PC membrane film at pH 7.4 (molar ratio: G1E peptide/egg PC ≈ 1/60).
Figure 4.13 The absorption (a) and second derivative (b) spectra of G1E peptide-egg PC membrane film at pH 7.4 hydrated by 4.4 μl D₂O for 5 hours (molar ratio: G1E peptide/egg PC = 1/60).
Figure 4.14 The absorption (a) and second derivative (b) spectra of dry G1E peptide-egg PC membrane film at pH 5 (molar ratio: G1E peptide/egg PC ≈ 1/60).
Figure 4.15 The absorption (a) and second derivative (b) spectra of G1E peptide-egg
PC membrane film at pH 5 hydrated by 94 µl D$_2$O+DCI (pD 5) for 3 hours (molar
ratio: G1E peptide/egg PC = 1/60).
4.3.2.4. *HIV-1 peptide in lipid membranes.*

HIV-1 peptide was reconstituted into egg PC lipid membrane in the same way as other peptides, then hydrated with distilled water to form suspensions. However, since HIV-1 peptide is very hydrophobic, when higher peptide/lipid molar ratio was used, the HIV1 peptide molecules aggregated to form films which were very difficult to hydrate to form a suspension. Thus a low peptide/lipid molar ratio of 1/250 was used to prepare HIV1 peptide-egg PC suspension in order to reduce the chance of self-aggregation of the peptide. The absorption and second derivative spectra of dry HIV-1 peptide-egg PC film (molar ratio: HIV-1 peptide/egg PC = 1/250) are shown in Figure 4.16a and 4.16b. Two weak absorption bands can be seen at 1660 cm\(^{-1}\) and 1632 cm\(^{-1}\) in amide I band area (due to low peptide/lipid ratio). The amide II band is shown to be weak and can be hardly seen. The second derivative spectrum (Figure 4.16b) reveals several components in amide I band area: a band at 1692 cm\(^{-1}\), is assigned to \(\beta\)-turns or \(\beta\)-sheet; a weak shoulder at 1671 cm\(^{-1}\), is assigned to turns structure; a major band at 1657 cm\(^{-1}\), is assigned to \(\alpha\)-helix; and a band at 1629 cm\(^{-1}\), is assigned to \(\beta\)-sheet. Thus the secondary structure of HIV1 peptide in egg PC membrane was \(\alpha\)-helical with minor contributions from other structures, this is different from the results of Rafalski *et al.* (1991), which only observed the \(\beta\)-conformation of HIV N-terminal LAV\(_{1a}\) and LAV\(_{mal}\) peptides when these peptides interacted with neutral lipid vesicles.

The dry HIV-1 peptide-egg PC film was hydrated with 70 \(\mu\)l D\(_2\)O. The absorption and second derivative spectra of the film hydrated for 12 hours are given in Figure 4.17a and 4.17b. The second derivative spectrum reveals the presence of several components in amide I' band area: a weak band at 1674 cm\(^{-1}\), which was shifted from the band at 1692 cm\(^{-1}\) in the spectrum of dry film (Figure 4.16b) after hydration, is
Figure 4.16 The absorption (a) and second derivative (b) spectra of dry HIV1 peptide-egg PC membrane film (molar ratio: HIV1 peptide/egg PC ≈ 1/250).
Figure 4.17 The absorption (a) and second derivative (b) spectra of HIV1 peptide-egg PC membrane film hydrated by 70 μl D₂O for 12 hours (molar ratio: HIV1 peptide/egg PC ≈ 1/250).
assigned to turns structure; a band at 1652 cm\(^{-1}\), which was shifted from the band at 1657 cm\(^{-1}\) in Fig. 4.16b after hydration, is confirmed to be \(\alpha\)-helix; a band at 1625 cm\(^{-1}\), is assigned to \(\beta\)-sheet. The carbonyl group of egg PC was fully hydrated, as shown in Fig. 17b. Although there was some increase of the content of \(\beta\)-sheet structure after hydration, a certain amount of \(\alpha\)-helix still remained unchanged. It seems that at a low peptide/lipid ratio, some HIV-1 peptide molecules inserted into lipid membrane in the monomeric form. This may have enabled the peptide to remain in an \(\alpha\)-helix, otherwise it would aggregate to form a \(\beta\)-sheet when the sample was in the aqueous suspension before the sample was dried on the crystal surface to form dry film.

4.3.2.5. Polarised FT-IR studies

Polarised FT-IR measurements and the determination of dichroic ratio and orientation of several absorption bands were carried out as described in Appendix I. Typically, a dichroic ratio is the average of three measurements unless otherwise stated. The angle calculated from dichroic ratio is the angle between the molecular chain direction and the normal direction of the Ge crystal surface. To determine the orientation of a molecular chain relative to lipid membrane, the orientation of the lipid membrane should be also considered. Table 4.2 shows the dichroic ratio (R) of \(\alpha\)-helix of WT, G1E, HA, and HIV1 peptides in dry sample films. It appears that the \(\alpha\)-helices of these peptides were not inserted into lipid membranes fully perpendicularly. There are a minor but consistent difference between the orientation of the \(\alpha\)-helices of WT, G1E and HA peptides at neutral pH and their orientation at acidic pH. The \(\alpha\)-helices of WT, G1E and HA peptides in the lipid membranes at acidic pH are more oblique to the Ge crystal surface.
<table>
<thead>
<tr>
<th>α-helix</th>
<th>WT peptide in dry egg PC membranes</th>
<th>G1E peptide in dry egg PC membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 7.4</td>
<td>pH 5</td>
</tr>
<tr>
<td></td>
<td>pH 5</td>
<td>pH 7.4</td>
</tr>
<tr>
<td>Dichroic ratio R</td>
<td>1.54 ± 0.03</td>
<td>1.35 ± 0.02</td>
</tr>
<tr>
<td>θ</td>
<td>36°</td>
<td>44°</td>
</tr>
<tr>
<td>α-helix</td>
<td>HA peptide in dry egg PC membranes</td>
<td>HIV1 peptide in egg PC membranes</td>
</tr>
<tr>
<td></td>
<td>pH 7.4</td>
<td>pH 5</td>
</tr>
<tr>
<td></td>
<td>pH 5</td>
<td>pH 7.4</td>
</tr>
<tr>
<td>Dichroic ratio R</td>
<td>1.65 ± 0.08 (6)</td>
<td>1.31 ± 0.02 (4)</td>
</tr>
<tr>
<td>θ</td>
<td>33°</td>
<td>46°</td>
</tr>
</tbody>
</table>

Table 4.2 The dichroic ratio (R) of the α-helices of WT, G1E, HA and HIV1 peptides in dry sample films. The angles (θ) were calculated according to equation E.A.3 in Appendix I.

The dichroic ratios of the egg PC symmetric CH₂ stretching band (at ~ 2850 cm⁻¹) and the carbonyl band of egg PC (at ~ 1737 cm⁻¹) in dried samples are shown in Table 4.3 and Table 4.4, respectively. When no buffer was used, egg PC membranes were well-orientated, and the angle between the lipid acyl chains and the normal direction of the Ge crystal surface is 25°, which is in good agreement with other reports. It seems that if buffers were used, when the sample suspensions were dried on Ge crystal surface, the buffer substance disturbed the orientation of the lipid membranes, which is shown by the deviation of the lipid acyl chain direction from the lipid acyl chain direction of the well orientated egg PC membranes in which no buffer was used. However, Table 4.4 shows that the orientation of the carbonyl group of egg...
### Table 4.3

<table>
<thead>
<tr>
<th>Egg PC CH(_2) band (\sim 2850) cm(^{-1})</th>
<th>WT peptide in egg PC membranes</th>
<th>G1E peptide in egg PC membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 7.4</td>
<td>pH 7.4</td>
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<tr>
<td>Dichroic ratio (R)</td>
<td>1.62 ± 0.01</td>
<td>1.59 ± 0.03</td>
</tr>
<tr>
<td>(\theta)</td>
<td>45°</td>
<td>44°</td>
</tr>
<tr>
<td>pH 5</td>
<td>1.31 ± 0.07</td>
<td>1.37 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>35°</td>
<td>37°</td>
</tr>
<tr>
<td>Egg PC CH(_2) band (\sim 2850) cm(^{-1})</td>
<td>HA peptide in egg PC membranes</td>
<td>HIV1 peptide in egg PC membranes</td>
</tr>
<tr>
<td></td>
<td>pH 7.4</td>
<td>pH 7.4</td>
</tr>
<tr>
<td>Dichroic ratio (R)</td>
<td>1.62 ± 0.02 (6)</td>
<td>1.14 ± 0.02</td>
</tr>
<tr>
<td>(\theta)</td>
<td>45°</td>
<td>28°</td>
</tr>
<tr>
<td>pH 5</td>
<td>1.38 ± 0.06 (4)</td>
<td>1.08 (2)</td>
</tr>
<tr>
<td></td>
<td>38°</td>
<td>25°</td>
</tr>
</tbody>
</table>

Table 4.3 The dichroic ratios of the egg PC symmetric CH\(_2\) stretching band (at \(\sim 2850\) cm\(^{-1}\)) in dry samples. The angles \(\theta\) were calculated according to equation E.A.3 in Appendix I.

### Table 4.4

<table>
<thead>
<tr>
<th>Egg PC C=O band (\sim 1737) cm(^{-1})</th>
<th>WT peptide in egg PC membranes</th>
<th>G1E peptide in egg PC membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 7.4</td>
<td>pH 7.4</td>
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<tr>
<td>Dichroic ratio (R)</td>
<td>1.55 ± 0.01</td>
<td>1.55 ± 0.02</td>
</tr>
<tr>
<td>pH 5</td>
<td>1.44 ± 0.01</td>
<td>1.44 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>1.44 ± 0.01</td>
<td>1.44 ± 0.01</td>
</tr>
<tr>
<td>Egg PC C=O band (\sim 1737) cm(^{-1})</td>
<td>HA peptide in egg PC membranes</td>
<td>HIV1 peptide in egg PC membranes</td>
</tr>
<tr>
<td></td>
<td>pH 7.4</td>
<td>pH 7.4</td>
</tr>
<tr>
<td>Dichroic ratio (R)</td>
<td>1.58 ± 0.02 (6)</td>
<td>1.48 ± 0.02</td>
</tr>
<tr>
<td>pH 5</td>
<td>1.50 ± 0.03 (4)</td>
<td>1.48 (2)</td>
</tr>
<tr>
<td></td>
<td>1.48 ± 0.02</td>
<td>1.48 (2)</td>
</tr>
</tbody>
</table>

Table 4.4 The dichroic ratios of the egg PC carbonyl band (at \(\sim 1737\) cm\(^{-1}\)) in dry samples.
<table>
<thead>
<tr>
<th>Egg PC CH(_2) band ~ 2850 cm(^{-1})</th>
<th>WT peptide in egg PC membranes</th>
<th>G1E peptide in egg PC membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.4</td>
<td>pH 5</td>
<td>pH 7.4</td>
</tr>
<tr>
<td>(\theta)</td>
<td>40°</td>
<td>40°</td>
</tr>
<tr>
<td>pH 7.4</td>
<td>pH 5</td>
<td>HA peptide in egg PC membranes</td>
</tr>
<tr>
<td>(\theta)</td>
<td>38°</td>
<td>37°</td>
</tr>
</tbody>
</table>

**Table 4.5** The dichroic ratios of the egg PC symmetric CH\(_2\) stretching band (at ~2850 cm\(^{-1}\)) in hydrated samples. The angles (\(\theta\)) were calculated according to equation E.A.3 in Appendix I.

<table>
<thead>
<tr>
<th>(\alpha)-helix</th>
<th>WT peptide</th>
<th>G1E peptide</th>
<th>HA peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 7.4</td>
<td>pH 5</td>
<td>pH 7.4</td>
</tr>
<tr>
<td>Dichroic ratio R</td>
<td>1.56 ± 0.03</td>
<td>1.32 ± 0.02</td>
<td>1.53 ± 0.06</td>
</tr>
<tr>
<td>(\theta)</td>
<td>36°</td>
<td>46°</td>
<td>37°</td>
</tr>
</tbody>
</table>

**Table 4.6** The dichroic ratio (R) of the \(\alpha\)-helix in hydrated sample films. The angles (\(\theta\)) were calculated according to equation E.A.3 in Appendix I.

PC in the membrane was only affected very slightly by the using of buffers. Thus the deviation of the orientation of lipid membrane dried with buffer may be overestimated by only considering the orientation of the egg PC symmetric CH\(_2\) stretching band (at

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For dry egg PC membranes, the antisymmetric CH\textsubscript{2} band was at 2921 cm\textsuperscript{-1}, when the sample film was hydrated, the band shifted to 2924 cm\textsuperscript{-1}, which is suggested to be due to the melting of the hydrocarbon chains accompanied by the introduction of the gauche conformers after hydration (Asher & Levin, 1977). For dry sample films with Hepes, the antisymmetric CH\textsubscript{2} band was at 2924 cm\textsuperscript{-1}. After hydration, this band didn’t shift to higher frequency, which indicates that the gauche conformers existed in lipid acyl chains in the dry films with Hepes. This may explain why the deviation of lipid acyl chain direction was much bigger than the deviation of the direction of the lipid carbonyl group in the dry sample films with buffer substance.

After considering the difference of orientation of the lipid membranes between the dry sample films at neutral pH and the dry films at acidic pH, the difference of the orientation of the \(\alpha\)-helices of WT, G1E, HA peptides relative to the lipid membrane surface between neutral pH and acidic pH samples films would be only bigger, at least

<table>
<thead>
<tr>
<th>(\beta)-sheet</th>
<th>WT peptide in hydrated egg PC membranes</th>
<th>G1E peptide in hydrated egg PC membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 7.4</td>
<td>pH 5</td>
</tr>
<tr>
<td>Dichroic ratio</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>B-sheet</td>
<td>1.64 ± 0.01</td>
<td>1.67 ± 0.09</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(\beta)-sheet</th>
<th>HA peptide in hydrated egg PC membranes</th>
<th>HIV1 peptide in egg PC membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 7.4</td>
<td>pH 5</td>
</tr>
<tr>
<td>Dichroic ratio</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>B-sheet</td>
<td>1.65 ± 0.08</td>
<td>1.66 ± 0.09 (4)</td>
</tr>
</tbody>
</table>

Table 4.7 The dichroic ratio (R) of the \(\beta\)-sheet in hydrated sample films.
not smaller than the differences of their orientations relative to the normal direction of Ge crystal surface. The \( \alpha \)-helices of WT, G1E, and HA peptides in the lipid membranes at acidic pH thus are more oblique to the lipid membrane plane than at neutral pH.

Figure 4.18 shows the second derivative of polarised IR spectra of hydrated HA peptide-egg PC sample film (pD 7.4). It appears that the hydrated egg PC carbonyl groups (at \( \sim 1729 \) cm\(^{-1} \)) were more parallel to the lipid membrane plane than the free carbonyl groups (at \( \sim 1742 \) cm\(^{-1} \)), which were shown by the stronger hydrated carbonyl band in the spectrum recorded with vertical (0°) polarised radiation than in the spectrum recorded with parallel (90°) polarised radiation. This indicates that the carbonyl group of the hydrated egg PC membranes is well orientated. These phenomena were observed in all samples studied in the present work, so the sample lipid membrane layers prepared in the experiments reported here were well orientated in carbonyl group C=O position. This was confirmed when the dry sample films were hydrated, the difference of orientation of egg PC symmetric CH\(_2\) stretching band (at \( \sim 2850 \) cm\(^{-1} \)) in the peptides-lipid membranes observed between neutral pH and acidic pH disappeared (Table 4.5).

The observations of the orientations of \( \alpha \)-helices in dry WT, G1E and HA peptides-lipid membranes were confirmed by the polarization studies of the orientations of \( \alpha \)-helices of these peptides in hydrated sample films (Table 4.6). However, it should be noticed that, several possible errors may contribute to the polarization measurement of the hydrated sample films. After hydration, due to the formation of \( \beta \)-sheet, the \( \alpha \)-helices band (at \( \sim 1650 \) cm\(^{-1} \)) remained overlapped with the \( \beta \)-sheet band (at \( \sim 1626 \) cm\(^{-1} \)) in some extent. Although when the baseline determination method was applied to the spectra recorded with both parallel and vertical polarised light at the same time, which
Figure 4.18 The second derivative spectra of hydrated HA peptide-egg PC membrane film at pH 7.4 recorded with vertical (0°) polarised radiation (a) and parallel (90°) polarised radiation (b)(molar ratio: HA peptide/egg PC ≈ 1/60).
may compensate in certain extent of the errors due to the overlap of the \( \alpha \)-helices and \( \beta \)-sheet bands, it is not known how much errors are still remained uncompensated. For hydrated G1E peptide-lipid membranes at acidic pH, the majority of the \( \alpha \)-helices in dry sample films transformed to \( \beta \)-sheet structure, so the orientation of \( \alpha \)-helix measured in hydrated G1E peptide-lipid membranes may be quite different from the measurements of the dry sample films. Furthermore, if certain \( \text{H}_2\text{O} \) exists in the \( \text{D}_2\text{O} \) hydrated samples with a relative high hydrogen/deuterium (H/D) ratio, the \( \text{H}_2\text{O} \) band needs to be subtracted with a spectrum of \( \text{D}_2\text{O} + \text{H}_2\text{O} \) which has a similar H/D ratio. Errors may be introduced by the subtraction, but it is still much better than using only \( \text{H}_2\text{O} \) to hydrate the sample film, since there are more uncertainties involved in quantitative subtraction of \( \text{H}_2\text{O} \) band from the \( \text{H}_2\text{O} \) hydrated sample film spectrum. More efforts are needed to improve the polarization measurement of the \( \text{D}_2\text{O} \) hydrated sample films.

In HIV-1 peptide-egg PC dry membrane film, both symmetric \( \text{CH}_2 \) stretching vibration (at \( 2850 \text{ cm}^{-1} \)) and carbonyl group stretching vibration (at \( 1737 \text{ cm}^{-1} \)) of egg PC had a similar orientation to the egg PC membrane films (Table 4.3 and Table 4.4). The \( \alpha \)-helix of HIV1 peptide in the membrane adopt an oblique orientation to the lipid membrane surface, which is in agreement with the theoretical predictions (Brasseur et al., 1988), but different from the results of Martin et al. (1993), which showed the \( \alpha \)-helix of a 16 residues peptide corresponding to the N-terminal sequence of gp41 protein of strain HXB2 HIV virus was parallel to the lipid/water interface when it is interacting with LUV vesicles of PC/SM/Chol (2:1:1.5 molar ratio). When HIV1 peptide-lipid membranes were hydrated, the \( \alpha \)-helix band became broad and could not be distinguished from the absorption spectrum, thus the dichroic ratio of this band was
According to Equation E.A.3 (Appendix I), when \( R = -1.43 \) the angle between the \( \beta \)-sheet chain direction and the normal direction of the Ge crystal surface will be 90°. All \( \beta \)-sheet structures measured in the present work thus were parallel to the lipid membrane surface (Table 4.7). This is different from the observations of Martin et al. (1993), which showed the \( \beta \)-sheet of the 16-residue HIV HXB2 N-terminal peptide had no preferred orientation relative to the lipid membrane.

### 4.4 Discussion

The lipid-mixing assay of WT, G1E, HA, and HIV1 peptides showed certain relevance between the fusion of neutral lipid vesicles induced by these synthetic fusion peptides and the cellular or viral fusion induced by intact viruses, thus the study of the conformations of these synthetic peptides in model membrane systems may provide some use to understand the mechanism of the viral membrane fusion process. However, since many features of intact virus are not present in the model system, cautions should be paid to explain the results from a model system, and ideally, these experiments should be confirmed by other independent observations.

In the present study, the reconstitution of these peptides in lipid membranes is crucial to whether the structural studies could provide correct information. The observation of the predominantly \( \alpha \)-helical structure of WT, G1E, HA peptides suggests that these peptides were not aggregated when they were reconstituted in lipid membranes to form aqueous suspension. The observation that the structure of HIV1 peptide in lipid membranes was \( \alpha \)-helical but with small contribution of \( \beta \)-sheet suggests that at least some HIV-1 peptide molecules inserted into lipid membranes as monomeric
In an aqueous peptide-lipid suspension, since these peptides are all hydrophobic, if these peptides were not reconstituted into lipid membranes, it is likely these peptides will aggregate to form inter-molecular β-sheet. This was confirmed by an experiment in which HA peptide was added from its boric buffer stock solution (pH 9.2) to a egg PC sonicated vesicle suspension (pH 7.4 or pH 5) with a peptide/lipid molar ratio 1/22. The HA peptide could induce the fusion of the egg PC sonicated vesicles at pH 5, but not at pH 7.4. The Transmission FTIR studies of these two sample suspensions showed that the secondary structure of the HA peptide were predominantly β-sheet with minor α-helix in both suspensions (not shown).

The insertion of these peptides into lipid membranes was confirmed by the observations that the carbonyl group of egg PC in WT, G1E, and HA peptides-lipid membranes at pD 5 were all less hydrated, indicating these peptides at least inserted to the carbonyl group position of egg PC in the lipid membranes.

The formation of β-sheet after the WT, G1E, and HA peptides-lipid films were hydrated may raise the question that using ATR technique to study hydrated protein (peptide)-lipid membranes may create artifacts. However, since many observations have been made on various hydrated protein (peptide)-lipid membranes, and no formation of β-sheet was reported so far. To confirm the reliability of the technique, alamethicin was reconstituted with egg PC lipid to form aqueous alamethicin-egg PC suspension. The dry sample film was prepared and rehydrated same as WT, G1E, HA and HIV1 peptide samples, but no formation of β-sheet was observed (not shown), and the observed secondary structure of alamethicin in the hydrated sample film is consist with the transmission FTIR studies of alamethicin-PC aqueous suspension.
(Haris & Chapman, 1988).

Since sometimes as little as 4.4 µl D₂O was used to hydrate the dry sample film, the sample was merely "wet", there being no obvious aqueous layer. It is unlikely such little D₂O will wash away lipid molecules to leave peptide molecules to stick on the Ge crystal surface. It seems that when the sample films are rehydrated, the tight contact between lipid membrane layers may enable peptide molecules in the lipid membrane to interact with one another.

Whether there is formation of β-sheet structures depends on several factors. Proteins (or peptides) that are anchored at the surface of lipid membrane are more likely to interact with one another and aggregate, through β-sheet formation, than transmembrane proteins like alamethicin or glycophorin. The formation of β-sheets may also be governed by the intrinsic tendency of peptide or protein molecules to aggregate. It has been reported that an influenza HA2 N-terminal synthetic fusion peptide molecules aggregated in solution at pH 7, 5 and 11 (Wharton et al., 1988). It was suggested that this aggregation enabled the peptides to interact simultaneously with more than one liposome. Slepushkin et al. (1992) also reported a similar aggregation of HIV gp41 N-terminal peptides in PBS solutions and they suggested that the lipid intermixing induced by the peptides correlates with the potential of the peptides to form long filaments in aqueous solution.

Membrane fluidity may also play some role in influencing the formation of β-sheets. Although the carbonyl groups C=O of egg PC molecules in G1E peptide-lipid and WT peptide-lipid membrane were both less hydrated at pD 5 than pD 7.4, the acyl chains of egg PC molecules in G1E peptide-lipid membrane were more easily "melted", as was indicated by the readily decrease of the intensity and broadening of the lipid
anti-symmetric (~2920 cm\(^{-1}\)) and symmetric (~2850 cm\(^{-1}\)) CH\(_2\) stretching bands induced by hydration. The quicker and more extensive formation of \(\beta\)-sheet of G1E peptide molecules in the hydrated membrane at pH 5 may be due to the fact that the G1E peptide inserted into the lipid membrane less deeply because of its charged glutamic acid residue at the N-terminal position of the peptide. Thus the fluidity of the hydrated G1E peptide-lipid membrane at pH 5 may be higher than the hydrated WT (or HA) peptide-lipid membranes. The observation that more \(\beta\)-sheet was formed when HA peptide-lipid membrane was hydrated at pH 7.4 than pH 5 (Fig. 4.4a and Fig. 4.6a) was unexpected since at pH 5 the peptides should be less charged than at pH 7.4, which may assist the aggregation of the peptides. This may similarly be explained by that hydrated HA peptide-lipid membrane at pH 7.4 being more fluid, while the hydrated HA or WT peptides-lipid membranes are more rigid at pH 5.

Unlike the assignment of \(\beta\)-sheet band at ~1626 cm\(^{-1}\) and \(\alpha\)-helix band at ~1650 cm\(^{-1}\) (in D\(_2\)O), there are more uncertainties in the assignment of protein or peptide absorption bands between 1670 cm\(^{-1}\) and 1680 cm\(^{-1}\). The possible contamination by TFA in synthetic peptide also makes the assignment more complicated. Confirmation by studies of synthetic peptides from different sources seems necessary to avoid possible artifacts or false assignment. It is noticeable that in the present study, although WT, G1E, HA and HIV1 peptides are from different sources, a common feature of these peptides is the appearance of a band (1672-1675 cm\(^{-1}\)) in the spectra of all hydrated peptide-lipid membranes, which was shifted from a band at a higher frequency in dry sample films. While a pair of bands at ~1624 cm\(^{-1}\) and 1689 cm\(^{-1}\) (in D\(_2\)O) are usually assigned to anti-parallel inter-molecular \(\beta\)-sheet, the band around (1672-1675 cm\(^{-1}\)) should be assigned to turns structure (Jackson et al., 1989) if it is not due to TFA.
Since in the present study, there is no indication of contamination of TFA in HA peptide, and an extra purification procedure was used to remove possible contamination of TFA in HIV1 peptide, the band (1672-1675 cm⁻¹) in the hydrated HA or HIV-1 peptides-lipid membranes should be assigned to turns structure, while the band (1672-1675 cm⁻¹) in the hydrated WT or G1E peptides-lipid membranes should at least partially be assigned to turns structure (See Results).

After the peptide-lipid membranes in the present study were hydrated, the secondary structures of these peptides in lipid membranes were a mixture of α-helices and β-sheet. However, the infrared spectroscopy technique used cannot distinguish between a peptide molecule which consist of both α-helix and β-sheet structures and the existence of two subpopulations of peptides each of which contains 100% α-helices or 100% β-sheets. Although the latter possibility cannot be excluded completely without further evidence, the former is favoured for following reasons. The formation of β-sheet in all the hydrated sample films in the present study was a gradual process, and not a sharp increase with a plateau period. The gradual formation of β-sheet is more likely due to the interaction of peptide molecules which were anchored to the surface of lipid membranes. A 2%-30% sucrose gradient centrifugation was carried out to try to separate possible subpopulations of β-sheet and α-helices in an HIV1 peptide-membrane sample. In the fraction containing lipid vesicles, β-sheet was found to be still associated with the lipid membrane. The existence of turns structure in these peptides in lipid membranes may also provide an explanation for the co-existence of α-helix and β-sheet in the same peptide chain. In HA and HIV1 peptide-lipid membranes samples, since β-sheet existed before the rehydration process, it is possible that a small amount of peptide aggregated to form β-sheet. However, the increases of β-sheet content after
hydration were similar to WT and G1E peptides-lipid membranes sample and may share a common mechanism. Further evidence is needed, however, to resolve these possibilities.

One of the aims of the present study was to study several synthetic fusion peptides at the same time and try to find out the common feature which is necessary for peptide-induced membrane fusion processes. It seems no single factor is sufficient. The similar secondary structures of HA peptide in dry HA peptide-lipid membrane sample film at pH 7.4 and pH 5, and the similar secondary structures of WT peptide and G1E peptide in dry WT or G1E peptides-lipid membrane at both pH, indicated that the secondary structure of these peptides (at least as far as their content of α-helix is concerned) is not the only factor to influence the peptide-induced membrane fusion process. It has been assumed that it is necessary to overcome membrane hydration repulsion forces to induce membrane fusion. This has been confirmed by the observation that the carbonyl groups of egg PC in WT, G1E and HA peptide-lipid membranes at pD 5 were less hydrated than at pD 7.4. However, it also appears that an increase of hydrophobicity of the lipid membrane surface may not be sufficient to induce membrane fusion, since the G1E peptide could not induce membrane fusion at pH 5.

While a role for the α-helix in peptide-induced membrane fusion processes is favoured by many reports (Lear & DeGrado, 1987; Wharton et al., 1988; Takahashi, 1990; White, 1992), the possible role of β-sheet in inducing membrane aggregation (Rafalski et al., 1990) and lysis of membrane (Gordon et al., 1992) have also been suggested. Gallaher et al. (1992) has questioned the fusion peptide "sided" helical hypothesis. It has been noted that many fusion peptide sequences contain helix-disrupting (or breaking) glycine residues (Lear & DeGrado, 1987; Gallaher et al.,
including the peptides investigated in the present study. Lear & DeGrado (1987) also suggested that glycine residues appear to be important for stabilizing turns conformations. This all supports the idea that formation of \( \beta \)-sheet in the hydrated peptide-lipid membranes of the present study may reflect the intrinsic tendency of these peptide to "break" \( \alpha \)-helix and form inter-molecular \( \beta \)-sheet. BHA (bromelain-released influenza hemagglutinin) aggregates via its fusion peptide (Daniels et al., 1983). The inactivation of influenza virus, when incubated at low pH in the absence of target membranes (Sato et al., 1983a; Junankar & Cherry, 1986; Stegmann et al., 1987), is most likely caused by irreversible aggregation of viral HA trimers, involving hydrophobic interactions between adjacent fusion peptides (Stegmann et al., 1987; Junankar & Cherry, 1986). Thus the formation of inter-molecular \( \beta \)-sheets between fusion peptides offers a feasible molecular explanation for this virus inactivation process. Ramalho-Santos et al. (1993) suggested that influenza virus fusion activity and inactivation share a common mechanism. That the mobility of hemagglutinin is significant for its fusion activity was proposed by Junankar & Cherry (1986) and Gutman et al. (1993). The requirement that more than one HA trimer is needed to form a fusion complex for membrane fusion was suggested by Morris et al. (1989) and Ellens et al. (1990). The above several lines of evidence all support the concept that the formation of inter-molecular \( \beta \)-sheets between fusion peptides of different HA trimers to form a fusion complex in the influenza viral membrane fusion process is possible.

In addition to the observed minor but consistent differences of orientation of \( \alpha \)-helices of WT, G1E and HA peptide in peptide-lipid membranes between neutral and acidic pH, the more oblique orientation of the \( \alpha \)-helices of HIV1 peptide may also support the possibility that an oblique orientation of fusion peptides in lipid membranes
may be necessary for fusion activities. An oblique insertion of fusion peptides into target membranes may cause a bigger disruption to the target membrane that may be necessary for membrane fusion. The oblique orientation of fusion peptides in lipid membranes was supported by the finding that, the fusion active peptide showed a higher collapse pressure and a greater limiting molecular area when fusion peptides were spread at air-water interface (Burger et al., 1991). The failure of G1E peptide, and its corresponding influenza mutant virus, to induce membrane fusion may be because the fusion peptide cannot insert into target membrane. Subsequently it may aggregate with other adjacent peptides to form inter-molecular β-sheets which serve as ‘self-lock’ and lead to inactivation. The mechanism of inactivation of influenza virus when incubated at low pH in the absence of target membranes may be similar, with the formation of a 'self-lock' leading to inactivation. Since the fusion peptides of influenza virus hemagglutinin are not exposed at neutral pH, this may explain why there is no inactivation of influenza HA trimers at neutral pH, even though influenza N-terminal synthetic fusion peptide in lipid membranes may form β-sheet at neutral pH.

In conclusion it seems that, although individual factors such as the oblique insertion of α-helix into target membrane, the increase of hydrophobicity of the target membrane surface which is induced by the insertion of the fusion peptide, and the aggregation of fusion peptides or HA trimers, are all necessary to induce membrane fusion, the individual factors are not sufficient to induce membrane fusion. Only a combination of these factors to form a fusion complex is sufficient, and necessary, to induce membrane fusion. It is quite possible that, in the influenza viral HA induced membrane fusion process, fusion peptides bind and insert into lipid membranes obliquely through their α-helices and are subsequently aggregated through their β-sheet
4.5 Summary

The secondary structures and orientations of the fusion peptides (corresponding to the N-terminus of HA2 protein of strains A/PR/8/34 and X31 influenza viruses (HA and WT (wild type) peptides, respectively), a peptide (G1E peptide) with a substitution of glutamic acid for the glycine residue at the N-terminal of the WT peptide, and the fusion peptide corresponding to the N-terminus of gp41 protein of ARV2 strain HIV-1 virus were investigated in egg phosphatidylcholine bilayers with polarized, attenuated total reflection (ATR), Fourier transform infrared spectroscopy (FT-IR). Both dried and hydrated samples were studied.

Peptides-lipid membranes aqueous suspensions were dried on a germanium crystal. For each peptide studied, the α-helix was the most important secondary structure, but there was also evidence for contributions from β-sheet and turns structures.

The carbonyl groups of egg PC molecules in WT, G1E and HA peptides-lipid membranes were shown to be more less hydrated at pD 5 than at pD 7.4. Hydrogen-deuterium exchange experiments showed that the amide II band of these fusion peptides decreased greatly after deuteration, indicating that the fusion peptides were probably not deeply inserted into the lipid membrane bilayer.

After hydration with D₂O, there was a tendency for a transition from α-helix structure to β sheet structure in the influenza fusion peptides. This transition might be induced by contact between lipid membranes to which fusion peptides are anchored, and might depend on the fluidity of the lipid membrane. It seems that the fusion peptides
may move laterally to interact with one another to form aggregated $\beta$-sheet.

HIV1 peptide induced the fusion of sonicated egg PC liposomes. The secondary structure of HIV-1 peptide in the dry peptide-lipid membrane was a major $\alpha$-helix with a small contribution from $\beta$-sheet or turns structure. After hydration by $D_2O$, there was slight increase of the content of $\beta$-sheet in HIV1 peptide, but certain amount of $\alpha$-helix still remain unchanged. While $\alpha$-helix may play an important role in membrane fusion process, a possible role for $\beta$-sheet structures in the process also cannot be excluded.

The orientation of the $\alpha$-helix in WT, G1E, and HA peptides was not completely perpendicular to the lipid membrane plane, and was more oblique to the lipid membrane surface at pH 5 than at pH 7.4. The orientation of the $\alpha$-helix of HIV1 peptide was oblique relative to lipid membrane surface. The oblique insertion of $\alpha$-helix seems necessary for the disruption of the lipid membrane and membrane fusion. All $\beta$-sheets studied in the present work were parallel to the lipid membrane surface.

It seems that although individual factors such as the oblique insertion of $\alpha$-helix into target membrane, the increase of hydrophobicity of the target membrane surface which is induced by the insertion of the fusion peptide, and the aggregation of fusion peptides or HA trimers, are all necessary to induce membrane fusion, these individual factors are not sufficient to induce membrane fusion. Only a combination of these factors to form a fusion complex is sufficient, and necessary, to induce membrane fusion. It is quite possible that in the influenza viral HA induced membrane fusion process, fusion peptides bind and insert into lipid membranes obliquely through their $\alpha$-helices and are subsequently aggregated through their $\beta$-sheet structures.
5.1 Fusion Assay

It has been reported that the kinetics of different liposome fusion assays are not consistent with one another (Silvius et al., 1988; Düzgüneş et al., 1988). A probe-mixing assay and a probe-dilution assay (involving energy transfer between NBD-PE and rhodamine-PE), and contents mixing assays including the Tb/DPA (terbium/dipicolinic acid) assay and ANTS/DPX (aminonaphthalene-trisulfonate/xyylene-bis-pyrindinium bromide) assay, are most frequently used to assay liposome fusion. The fusion rates for a given liposome fusion process obtained by these assays are different, in the following sequence: probe mixing > probe dilution > ANTS/DPX > TB/DPA. There have been many comments on the disagreement between the different assays and many possible artifacts have been proposed (Silvius et al., 1988; Düzgüneş et al., 1988).

In our experiments, it was found that exposure of erythrocytes to electrical breakdown pulses was followed by the almost-instantaneous diffusion of carboxyfluorescein, whereas the fluorescent membrane probe Dil, diffused more slowly (especially at lower temperature). The cytoplasmic probe carboxyfluorescein is therefore more suitable to use to investigate the kinetics of cell fusion. The diffusion of membrane probes between fused labelled and unlabelled cells represents both membrane mixing and the diffusion of membrane probe in lipid or cell membranes. The latter may be the rate limiting step in fusion assays and is also influenced by temperature. Consequently, artifacts may be introduced when membrane probes are used to
investigate the kinetics of cell fusions and liposome fusions. The fact that the contents mixing assays of liposome fusion using Tb/DPA or ANTS/DPX probes gave slower kinetics than membrane mixing assay may be due to mixing and reaction between terbium ions and dipicolinic acid or ANTS and DPX being the rate limiting step. These assays are therefore not reliable when they are used to investigate the kinetic of liposome fusion.

The membrane mixing and content mixing assays can be used to investigate the extent of liposome fusion after the diffusion of both kind of probes reach equilibrium. While membrane mixing assays are stable after the diffusion of membrane probes reach equilibrium, care may need be taken to allow for the possible leakage of cytoplasmic content probes. When considering possible hemi-fusion indicated by membrane mixing assay, and the leakage of cytoplasmic content probes, a combination of both membrane mixing and contents mixing assays of liposome fusion is most preferable.

For liposome fusion with rapid leakage, it is impossible to use a contents mixing assay. In this case, membrane mixing assays could still be used to investigate liposome fusions qualitatively. The hemi-fusion found in liposome fusion assays and in the present experiments may be treated as another kind of fusion: outer layer membrane fusion, and not an artifact of fusion assays. For cell fusion assays, the cytoplasmic probe is suitable to investigate cytoplasmic fusion, while the membrane probe is suitable to investigate plasma membrane fusion.

5.2 Osmotic forces and membrane fusion

It was suggested that osmotic forces act to supplement the electrical compressive force in the fusion model proposed by Lucy and Ahkong (1986). Electrofusion can be induced without osmotic forces as long as the field strength and the duration time of the
fusion pulse are high and long enough. Osmotic forces may stretch the membrane and the cell cytoskeleton before, during, or after the application of breakdown pulses. However, Schmitt and Zimmermann (1989) believed that the osmotic pressure gradient between the attached cells should always be zero or at least negligible. They observed that the fusion and hybrid yields remained high or exhibited even higher values when the cells were returned from hypo-osmolar to iso-osmolar conditions before electrofusion, so they concluded that neither pressure nor osmotic gradients, nor the induced increase of cell volume (including the associated tensions in the membrane) are per se responsible for the fusion of attached membranes. Furthermore, Schmitt and Zimmermann (1989) suggested that results which showed that electrofusion of myeloma cells with lymphocytes or other mammalian cells in hypo-osmolar solutions could not enhance the yield of hybrids should be considered as evidence to prove that osmotic forces cannot supplement the electrical compressive forces in electrofusion.

In fact, if the osmotic forces are too strong, they may increase lysis and reduce the viability of fused cells. Thus there should be a limited range in which osmotic forces can increase the incidence of electrically induced cell fusion. The decrease in production of hybridoma cells resulting from too strong osmotic forces need not therefore be considered as evidence against the possible role of osmotic forces in supplementing the electrical compressive force in electrofusion. Furthermore, the production of hybridoma cells involves many factors, for example, the pairing of myeloma cells and lymphocytes, and the viability of fused cells, it seems that this system is not suitable to investigate electrically induced cell fusion quantitatively.

Schmitt and Zimmermann (1989) also proposed that the increase in size of cells in hypo-osmolar solution led to an inappropriately high field strength of fusion pulse,
and suggested that the field strength of fusion pulse should be corrected according to the change in size of cells. However, in the work of present thesis, with erythrocytes in solutions having the same osmolarity but containing sugars of different sizes, the rounding up of electrofused erythrocytes was related closely to swelling of the pulsed cells, which resulted from the entering of these sugar molecules through electropores. Since the size of cells in solutions with same osmolarity are similar, the differences in rounding-up after pulsation arose from differences in cell swelling in these solutions, i.e. from the different osmotic forces experienced by the pulsed cells.

For a cell monolayer, or over grown cell layers in a culture plate, cell swelling could assist tight contact between cells. By contrast, when dielectrophoresis is used to bring cells in close contact, osmotic forces may not play a role in the cell contact process. However, it may still have a role in influencing cytoskeleton system. Thus, osmotic forces may play a role before, during or after the electric pulsation.

5.3 Future Work

Although in the present study, both the procoagulant activity assay and the interaction of divalent cations with the pulsed cells suggested that phosphatidylserine molecules were exposed at the outer surface of the pulsed erythrocytes, it is necessary and important to use other assays to confirm or check the present results. In view of the change of erythrocyte membrane lipid asymmetry after pulsation and the role of osmotic swelling in the rounding-up of the electrofused erythrocytes, it is now desirable to investigate a possibly important role of the cytoskeleton in cellular fusion processes and in maintaining lipid asymmetry.

In viral fusion peptides studies, it is necessary to confirm whether an oblique insertion of fusion peptides is required with other fusion peptides. Further
improvements of polarised FTIR are also needed especially for samples with which the pH needs to be controlled. A combination of differently focussed investigations will be necessary to finally understand viral fusion mechanisms.
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ADDENDUM


PUBLICATIONS


Appendix I

Fourier transform infrared spectroscopy

A.1.1 Basic mechanism

A.1.1.1 Vibrational spectroscopy

Infrared technique is based on the interaction of infrared radiation with the vibrational modes of the chemical bond of molecules. The infrared region of the electromagnetic spectrum extends from approximately 0.75 to 500 \( \mu m \). Wavenumber (or reciprocal wavelength) is more commonly used. A molecule which has \( n \) atoms has \( 3n-6 \) possible different vibrations. Those vibrations have different vibrational energy levels. The types of vibrations that occur in a three atom molecule is shown in Figure A.1.

![Vibrations of a three-atom molecule.](image)

Figure A.1 Vibrations of a three-atom molecule.
Photons whose energy is equal to the difference between two vibrational energy levels of a chemical bond can be absorbed, exciting the bond from one vibrational energy level to another. Only those vibrations which result in a periodic change in the dipole moment for a given bond can be activated by the absorption of infrared radiation. These vibration are said to be infrared-active. The fundamental molecular rotation-vibration transitions are induced by absorbance in the 4000-400 wavenumber (cm⁻¹) range.

A.1.1.2 Dispersive and Fourier transform infrared spectrometers

A dispersive or conventional infrared spectrometer comprises of an IR source, a monochromator and a detector. Radiation of a narrow range of frequencies, selected by the monochromator and a series of slits, interacts with the sample of interest. The spectral region of interest can be scanned systematically.

A Fourier transform infrared spectrometer comprises of an IR source, an interferometer, a detector, and a computer with which fast Fourier transform can be carried out. In the interferometer, infrared radiation from the IR source is separated into two beams by a beamsplitter, one beam (50%) is reflected to a stationary mirror, another beam (50%) is transmitted to a moving mirror. After reflection, the waves

Figure A.2 Schematic representation of the Michelson interferometer.

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recombine and interact with the sample, and are then detected by the detector as an interferogram only if the mirror is moving (Figure A.2). The interferograms are stored in the computer. They then undergo an inverse Fourier transform to generate an infrared spectrum.

As the interferogram collected by a Fourier transform infrared spectrometer contains the information on all frequencies incident on the sample, one scan of FTIR spectrometer can generate an infrared spectrum (≤ seconds). It is much faster than a dispersive infrared spectrometer and multiple scans can be collected to increase the signal/noise ratio (s/n ratio) of the infrared spectrum. Other advantages of a FTIR spectrometer include: High energy throughput of FTIR spectrometer can increase the s/n ratio of infrared spectra and frequency precision of a FTIR spectrometer is improved by using a helium-neon laser for internal frequency calibration.

A.1.1.3 Resolution enhancement

Many spectral absorption bands appear as a broad band envelope resulting from the overlap of two or more broad bands which are centred close each other. The instrument may be unable to resolve them. Two mathematical techniques, deconvolution and second derivation, have been developed to narrow the widths of the component bands artificially without changing their frequency positions. They permit accurate measurement of the component frequencies to be made.

A.1.2 Applications in studying the structure and function of biomembranes

A.1.2.1 Lipids

Many groups within the lipid molecule are infrared active and lipids have been widely studied by infrared spectroscopy. Table A.1 shows the IR absorption frequencies of lipids. The PO\(^2\) stretching vibrations are sensitive to hydration. Lipid phase
Table A.1 The IR absorption frequencies of lipids.

<table>
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<tr>
<th>IR BAND POSITION (CM⁻¹)</th>
<th>ASSIGNMENT</th>
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<td><strong>ACYL CHAIN GROUPS</strong></td>
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<tr>
<td>2956</td>
<td>CH₃ asymmetric stretch</td>
</tr>
<tr>
<td>2920</td>
<td>CH₂ asymmetric stretch</td>
</tr>
<tr>
<td>2870</td>
<td>CH₃ symmetric stretch</td>
</tr>
<tr>
<td>2850</td>
<td>CH₂ symmetric stretch</td>
</tr>
<tr>
<td>1470</td>
<td>CH₂ bending/scissoring</td>
</tr>
<tr>
<td>1380</td>
<td>CH₂ symmetric deformation</td>
</tr>
<tr>
<td>1380-1190</td>
<td>CH₂ wagging band progression</td>
</tr>
<tr>
<td>1150-700</td>
<td>CH₂ rocking band progression</td>
</tr>
<tr>
<td><strong>HEADGROUPS</strong></td>
<td></td>
</tr>
<tr>
<td>3050</td>
<td>CH₃ asymmetric stretch of N(CH₃)₃</td>
</tr>
<tr>
<td>1740-1720</td>
<td>C —— O ester stretch</td>
</tr>
<tr>
<td>1490</td>
<td>CH₃ asymmetric bend of N(CH₃)₃</td>
</tr>
<tr>
<td>1405</td>
<td>CH₃ symmetric bend of N(CH₃)₃</td>
</tr>
<tr>
<td>1250</td>
<td>O — P = O asymmetric stretch</td>
</tr>
<tr>
<td>1200-1000</td>
<td>C — O stretch</td>
</tr>
<tr>
<td>1170</td>
<td>C — O from esters</td>
</tr>
<tr>
<td>1085</td>
<td>O — P = O symmetric stretch</td>
</tr>
<tr>
<td>1070</td>
<td>C — O from esters</td>
</tr>
<tr>
<td>1040</td>
<td>C — N stretch</td>
</tr>
<tr>
<td>900-800</td>
<td>P — O stretch</td>
</tr>
<tr>
<td>800</td>
<td>C — N stretch</td>
</tr>
</tbody>
</table>
transition can be monitored from the shift of the CH\textsubscript{2} asymmetric stretching vibration, from 2919 cm\textsuperscript{-1} to 2924 cm\textsuperscript{-1} as the temperature is raised through T\textsubscript{c}.

The carbonyl groups (C=O) of the lipid molecule are also sensitive to hydration and phase. The lipid carbonyl absorption has been shown by resolution enhancement techniques to comprise of two components at 1743 cm\textsuperscript{-1} and 1728 cm\textsuperscript{-1}. FTIR studies on \textsuperscript{18}O-substituted lipids suggest that the two bands arise from the C=O groups are at different hydration levels (Blume et al., 1988). As the temperature of hydrated lipid sample rises through T\textsubscript{c}, the intensity of the 1728 cm\textsuperscript{-1} increases, indicating a higher level of hydration in the liquid-crystalline phase. This results in a shift to a lower frequency of the lipid C=O absorption.

A.1.2.2 Proteins

Within proteins the important infrared active vibrations are the amide vibrations, shown in Table A.2. Each of the amide vibrations arises from the peptide bonds in the protein backbone. The most useful amide vibrations are the amide I and amide II vibrations. Each secondary structure of proteins has its own distinct hydrogen-bonding pattern between the oxygen of the C=O group and the hydrogen of the N-H group of the polypeptide backbone. The hydrogen bonds are strong enough to modify the C=O vibration. The amide I vibration is very sensitive to protein secondary structure as this vibration is arising from the C=O stretching (80 \%) vibration with a weak coupling from the N-H bending (10 \%) and C-N stretching (10 \%) vibrations, respectively (Miyazawa, 1960). The positions of the amide I vibrational absorption bands vary with different individual secondary structure of proteins. The FTIR spectroscopy studies carried out to date have led to the deduction of characteristic amide frequencies for various secondary structures (Table A.3).
<table>
<thead>
<tr>
<th>AMIDE VIBRATION</th>
<th>FREQUENCY (CM⁻¹)</th>
<th>ASSIGNMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3300</td>
<td>N —— H (s)</td>
</tr>
<tr>
<td>B</td>
<td>3100</td>
<td>N —— H (s)</td>
</tr>
<tr>
<td>I</td>
<td>1680-1600</td>
<td>80% C —— O (s)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10% N —— H (b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10% C —— N (s)</td>
</tr>
<tr>
<td>II</td>
<td>1580-1480</td>
<td>60% N —— H (b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40% C —— N (s)</td>
</tr>
<tr>
<td>III</td>
<td>1300-1230</td>
<td>30% C —— N (s)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30% N —— H (b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10% C —— O (s)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10% O —— C —— N (b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20% others</td>
</tr>
<tr>
<td>IV</td>
<td>770-626</td>
<td>40% O —— C —— N (b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60% others</td>
</tr>
<tr>
<td>V</td>
<td>800-640</td>
<td>N —— H (b)</td>
</tr>
<tr>
<td>VI</td>
<td>605-540</td>
<td>C —— O (b)</td>
</tr>
<tr>
<td>VII</td>
<td>200</td>
<td>C —— N (t)</td>
</tr>
</tbody>
</table>

s = stretch; b = bending; t = torsion

Table A.2 The IR absorption frequencies of proteins.
<table>
<thead>
<tr>
<th>Band positions (cm$^{-1}$)</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1630-40</td>
<td>$\beta$-sheet</td>
</tr>
<tr>
<td>1644</td>
<td>random</td>
</tr>
<tr>
<td>1650-55</td>
<td>$\alpha$-helix</td>
</tr>
<tr>
<td>1670-80</td>
<td>turns</td>
</tr>
<tr>
<td>1680-90</td>
<td>$\beta$-sheet</td>
</tr>
</tbody>
</table>

Table A.3 Band assignment for various protein secondary structures in D$_2$O.

The amide II vibration arises mainly from the N-H bending vibration, it is highly sensitive to hydrogen-deuterium exchange and can provide information concerning solvent accessibility of the protein.

A.1.2.3 Sampling Techniques

The spectra of H$_2$O, D$_2$O, and water vapour are shown in Fig. A.3. The strong H-O-H bending vibration band overlaps with the amide I band of proteins. For transmission measurement, this limits the sample thickness to 6 $\mu$m, and requires the sample solutions containing 20-50 mg/ml protein to obtain an adequate s/n ratio. For lipid-protein samples in aqueous media, the protein concentration is limited by the lipid concentration if protein/lipid ratio is low. Water bands can be digitally subtracted from the sample spectra to obtain protein amide I band spectra. As the D-O-D absorption frequency is found about 400 cm$^{-1}$ lower (Figure A.3), longer pathlengths (50 $\mu$m) can be used for samples in D$_2$O for transmission measurements. This increases
Figure A.3 The absorption spectra of $\text{H}_2\text{O}$, $\text{D}_2\text{O}$ and water vapour.
the s/n ratio greatly. Subtraction of D$_2$O is not really necessary for samples in D$_2$O or D$_2$O+H$_2$O with a high D/H ratio (H$_2$O may be generated from hydrogen-deuterium exchange). However, for samples in D$_2$O+H$_2$O with a low D/H ratio, the H$_2$O band in the sample spectrum needs be subtracted with a D$_2$O+H$_2$O spectrum which has a similar D/H ratio.

Water vapour bands also overlap with the protein amide I band. The major noise in infrared spectra is from water vapour. This is particularly apparent when band narrowing techniques, such as deconvolution and second derivation, are used, as these enhance the sharp water vapour bands. During the measurement, the spectrometer is purged by a stream of dried, air or nitrogen to get rid of water vapour. Water vapour bands in the sample spectra can be further subtracted using a pre-recorded water vapour spectrum.

Samples such as biological membranes, lipid-peptide or lipid-protein membranes in aqueous media, can be dried on a crystal surface to form thin films, and studied by

Figure A.4 Set-up for attenuated total reflection (ATR) spectroscopy. $E_1$ and $E_\perp$ are the parallel and perpendicular polarised components of the electric field of the incident beam. $E_x$, $E_y$, and $E_z$ are the field components with respect to the coordinate system corresponding to the reflection plate.

Attenuated Internal Reflection (ATR) infrared spectroscopy (Figure A.4). A detailed
description of this technique is provided by Harrick (1967). Total internal reflection will occur when an infrared radiation propagating in an infrared transparent crystal is incident at the boundary of the crystal and external medium, with the angle of incidence, $\theta > \cos^{-1}(n_2/n_1)$, where $n_1$ and $n_2$ are the refractive indices of the internal and external media, respectively. Total internal reflection only occurs when the refractive index of the crystal is higher than that of the external medium. An electromagnetic field exists in the rarer medium beyond the reflecting interface, even under conditions of total reflection. This field exhibits the frequency of the incoming light, but the amplitude falls off exponentially with distance $z$ from the surface,

$$E = E_0 e^{-zd_p}$$  \hspace{1cm} (E.A.1)

where $d_p$ denotes the depth of penetration and is given by

$$d_p = \frac{\lambda/n_1}{2\pi (\sin^2 \theta - n_{21}^2)^{\frac{1}{2}}}$$  \hspace{1cm} (E.A.2)

where $n_{21}$ stands for the ratio $n_2/n_1$, and $\lambda$ is the wavelength of infrared radiation in vacuum.

The infrared beams propagating in the crystal are reflected between two surfaces between 6-36 times, determined by the dimensions of the crystal. This allows the IR beam to interact repeatedly with the sample films deposited on the crystal surface, thereby enhancing the s/n ratio (Fig A.4). High quality spectra may thus be produced from as little as 30 $\mu$g of the dried sample.

When samples of biological membranes, lipid-protein or lipid-peptide membranes and liposome vesicles in aqueous are dried down on the surface of a crystal,
the large fragments of membranes or liposomes vesicles will be broken down into smaller membrane fragments, but still keep the original membrane structure and orientation of lipid molecules in the membrane.

Sample films deposited on crystal surface can be rehydrated by buffers, H$_2$O, and D$_2$O. As the penetration depth of infrared radiation beyond the crystal is only the order of magnitude of the wavelength $\lambda/n_1$, the ATR measurements of a hydrated sample film are less impeded by water absorption than transmission experiments, thus producing higher s/n ratio spectra with less quantity of samples than transmission measurements. However, more material is needed for hydrated sample films than dried sample films due to the broadening and decreasing intensity of the absorption bands, and the possible looser contact between the sample films and crystal surface after the hydration.

Rath et al. (1991) studied the structure of thermolytic COOH-terminal channel forming peptide of colicin E1 in hydrated films on AgCl windows which was formed by drying the aqueous suspensions of colicin E1 reconstituted with DMPC on the windows and was rehydrated by water saturated air with FTIR spectroscopy. The structure of the membrane-bound form of the colicin E1 channel forming peptide fragment was found to be predominantly $\alpha$-helical, which was very similar to the structure of the soluble thermolytic fragment of colicin E1. Baenziger et al. (1992) dried native, alkaline-extracted nAchR (nicotine acetylcholine receptor) membranes on either a plastic microscope coverslip or germanium internal reflection crystal and then equilibrated with flowing buffer. The drying procedure has no effect on the functional state of the nAchR. The films adhered strongly to either support even with buffer flowing continuously past the film surface.
The conformational studies of hydrated protein-lipid or peptide-lipid membranes films on an ATR crystal or other windows with FTIR spectroscopy have been carried out by many laboratories (Gremlich et al., 1983; Fringeli et al., 1986, 1989; Goormaghtigh et al., 1990; Rath et al., 1991; Baenziger et al., 1992; Martin et al., 1993). Rath et al. (1992) summarised several methods developed for depositing thin, orientated multilamellar films on planar supports. Determination of protein structures by transmission in solution and attenuated total reflection methods yielded similar results, indicating that the drying-rehydration cycle experienced by the sample on the ATR plate did not significantly modify the secondary structure of the protein (Martin et al., 1993).

A.1.3 Polarized IR

The ATR technique can be extended to polarized ATR infrared technique to measure the dichroic ratio R and determine the orientation of lipids and proteins in membranes deposited on the crystal surface. The dichroic ratio R of a given infrared absorption band is defined as the ratio of the absorption of the infrared radiation polarized parallel to the plane of incidence (A∥) to that polarized perpendicular to the plane of incidence (A⊥).

A.1.3.1 The experimental set-up and the coordinate system of polarized ATR IR

A polarizer is used to produce polarized infrared radiation (Fringeli and Günthard, 1981). Figure A.4 shows the experimental set-up schematically. θ is the angle of incidence; E∥ and E⊥ are parallel and perpendicular polarized components of the electric field of incident light, respectively; Ex, Ey, and Ez, are electric field components with respect to the coordinate system corresponding to the internal reflection plate (E∥ → Ex, Ez, E⊥ → Ey) (Kopp et al., 1975).
For membrane layers the molecular orientation is not distributed statistically. Most often one of the molecular axis is symmetrically distributed around one spaced-fixed direction. An uniaxial model with fiber-type orientation is assumed (Fraser, 1953). The z axis is the fiber axis for this uniaxial distribution. The c axis coincides with the molecular chain (rod axis) direction. The direction of the c axis is related to the z axis by a polar angle $\theta$.

**A.1.3.2 The angle between the transition moment of a vibration and its molecular chain direction**

$\alpha$ is the angle the transition moment associated with a particular vibration makes with the c axis (the molecular chain direction). The assumption is that all directions about the c-axis are equally probable. The $\alpha$ values for some lipid molecule vibrations are: 0° for ester carbonyl group $\text{C}=\text{O}$, 90° for symmetric and antisymmetric $\text{CH}_2$ stretching vibration of lipid acyl hydrocarbon chains (at $\sim 2850 \text{ cm}^{-1}$ and $\sim 2921 \text{ cm}^{-1}$ respectively). As the amide I vibration of protein predominantly consist of $\text{C}=\text{O}$ stretching vibration (80%), the direction of hydrogen-bondings ($\text{C}=\text{O} \cdots \text{H-N}$) within a protein molecule can be determined from the measurement and analysis of the dichroic ratio $R$ of the protein amide I band. The direction of the transition moment associated with a particular protein secondary structure amide I vibration is same as the direction of the hydrogen-bonding of this secondary structure. The angle $\alpha$ between the transition moment of a $\alpha$ helix amide I vibration and its molecular chain direction has been estimated as 27° (Rothschild & Clark, 1979). For $\beta$ sheet structures, the angle $\alpha$ has been estimated as 90° (Gremlich et al., 1983).

**A.1.3.3 The formulas of dichroic ratio $R$**

The dichroic ratio $R$ is given by Brauner et al. (1987) and Hübner and Mantsch
(91) in E.A.3.

\[ R^a = \frac{A_1}{A_2} = \frac{E_x^2}{E_y^2} \frac{E_z^2[(\frac{1}{2})\sin^2 \alpha - (1/3)(1 - \beta)]}{(\frac{1}{2})\sin^2 \alpha + (1/3)(1 - \beta)} \]  

(E.A.3)

where \( f \) is an order parameter. For thin sample films (thin compared with the penetration depth of the evanescent wave), the electric field amplitudes \( E_x, E_y, \) and \( E_z \) can be determined as

\[ E_y = E_z = \frac{2\cos \theta}{(1 - n_{31}^2)^{\frac{1}{2}}} \]

and for thick sample films (thick compared with the penetration depth of the evanescent wave),

\[ E_y = E_z = \frac{2\cos \theta n_{32}^2 \sin \theta}{(1 - n_{31}^2)^{\frac{1}{2}}[(1 + n_{31}^2)\sin^2 \theta - n_{31}^2]^\frac{1}{2}} \]

(1 - \( n_i \)) denotes the ratio of refractive indices \( n_i/n_k \) of medium \( i \) and \( k \), where \( i,k = 1,2 \) and 3 stand for ATR plate, sample films, and surrounding medium, respectively. For thick
sample films index 3 must be replaced by 2, thus leading to the equations E.A.5.

From the order parameter \( f \), and an uniaxial orientation of the fiber axis \( c \) with the normal of the ATR crystal plane, we are able to calculate the mean angle \( \theta \) between the macroscopic \( z \)-axis and the fiber axis \( c \) as

\[
f(\theta) = 0.5 \cdot (3 \cos^2 \theta - 1) \quad \text{(E.A.6)}
\]

**A.1.3.4 The thickness of sample films**

When a germanium ATR crystal (50 x 20 x 2 mm, face angle \( \vartheta : 45^\circ \)) is used, \( n_1 = 4 \) (crystal), \( n_2 = 1.44 \) (sample films), \( n_3 = 1 \) (air). The penetration depth \( d_p = 0.0654 \lambda \) (E.A.2). For an infrared radiation at 1650 cm\(^{-1} \), \( d_p = 0.4 \mu m \). For an infrared radiation at \( \sim 2920 \) cm\(^{-1} \), \( d_p \sim 0.22 \) \( \mu m \). If thin films are studied, \( E_x = 1.41 \), \( E_y = 1.46 \), and \( E_z = 0.728 \) (E.1.4) (Brauner, 1987). If thick films are studied, \( E_x = 1.398 \), \( E_y = 1.516 \), and \( E_z = 1.625 \) (E.A.5) (Hübner & Mantsch, 1991).

Ideally, for dried thin sample films, the thickness of the films should be \( \leq 0.1 \) \( \mu m \), so equation E.A.4 can be used for vibrational bands from 2920 cm\(^{-1} \) (antisymmetric CH\(_2\) stretching band) to 970 cm\(^{-1} \) (asymmetric N\(^+\) - (CH\(_3\))\(_3\) stretching band). For dried thick sample films, the thickness of the films should be \( \geq 1.4 \mu m \), so equation E.A.5 can be used for all vibrational bands.

However, for hydrated sample films, spectra with very poor s/n ratio will be recorded from very thin sample films (\( \leq 0.1 \mu m \)), which make it impossible to use any resolution enhancement (deconvolution or secondary derivation) techniques to analyze the spectra. For thick sample films (\( \geq 1.4 \mu m \)), it is not easy to hydrate the films properly with 10 \( \mu l \) - 50 \( \mu l \) aqueous buffer. In other hand a bulk aqueous buffer may wash off the sample films from the crystal surface, and results in spectra with poor s/n ratio too. A thick sample film requires more sample than a thin sample film, and this
is another factor has to be considered when the amount of some sample is low (for example, less than 10 mg).

The compromise of several factors is required to prepare a sample film with a medium thickness. For example, when 80 \( \mu l \) egg PC (\( M_r \approx 786 \)) lipid vesicles (1.25mg/ml) are deposited on germanium crystal surface in \(~3.2\ \text{cm}^2\), the thickness of the sample film can be estimated as \(~0.24\ \mu\text{m}\) and it can be considered as a thin film for protein amide I band (around 1650 cm\(^{-1}\)), thus equation E.A.4 can be used for amide I band. For lipid antisymmetric and symmetric CH\(_2\) stretching bands (around 2921 cm\(^{-1}\) and 2850 cm\(^{-1}\) respectively), the sample film with 0.24 \( \mu\text{m} \) thickness cannot be considered as a thin film (\( d_p \approx 0.22 \mu\text{m} \)), so equation E.A.4 cannot be used, however, it can be considered as a thick film for this band, and equation E.A.5 can be used for these bands. The orientation of sample lipid membranes (including lipid-protein membranes) can be determined from the dichroic ratio \( R \) of lipid symmetric CH\(_2\) stretching band (\(~2850\ \text{cm}^{-1}\)) and ester carbonyl (C=O) band (\(~1740\ \text{cm}^{-1}\)).

A.1.3.5 The determination of dichroic ratio \( R \) for a particular component band

Usually the protein amide I band has a complex band envelope that comprises several overlapped components. To calculate the dichroic ratio \( R \) for a particular component, the main problem has been in trying to accurately determine the ratio of the area of a component band in the spectrum recorded with parallel incidence polarized infrared radiation to the area of the same component band in the spectrum recorded with vertical incidence polarized infrared radiation. Several methods have been developed to deal with this problem.

*Curve-fitting* method relies on resolution enhancement analysis to identify the positions of the component bands of a complex band envelope. A synthetic curve can
be generated by positioning component bands at the previously determined band positions identified by deconvolution and second derivation and varying the relative intensities, widths and band shapes (Lorentzian, Gaussian, or various combinations of the two) of the bands based upon least squares analysis until the synthetic curve matches the experimentally obtained complex band envelope (Susi & Byler, 1986). The area of each component band is then calculated and the dichroic ratio $R$ can be determined.

Although curve-fitting has been widely used, it relies on a number of assumptions:

1) It assumes that all component bands have the same shape. In fact they are not all the same for complex molecules.

2) It assumes that all component bands are symmetrical. They are not for complex molecules.

3) It assumes that all the component bands within the complex band envelope are known. We only know a minimum number of those component bands from resolution enhancement analysis.

The above assumptions raise a number of serious questions over the above method.

**Base line determination** is an alternative method to calculate the dichroic ratio $R$ for a particular component band. This method can be only used in very limited cases. To determine a baseline of a component band, two minimum points are selected on either side of the band. These bands are set as zero intensity. The intensity of this band with this baseline thus can be determined. The dichroic ratio $R$ of this component band will be the ratio of the intensities of this band at parallel polarization ($90^\circ$) to the same band at vertical polarization ($0^\circ$).
The ratio of the area of a band at parallel and vertical polarization is not always equal to the ratio of the intensity of this band at parallel and vertical polarization.

1) Only when this band is isolated enough like the symmetric stretching band of lipid acyl chain CH$_2$ (2850 cm$^{-1}$), then this method is valid.

2) When a complex band is predominately dominated by one single secondary structure, for example, a strong $\alpha$-helix (1650-1660 cm$^{-1}$) with a few weak bands at certain wavenumbers away, like a weak $\beta$-sheet band ($\sim$1690 cm$^{-1}$), the baseline determination method can be used with the ignorance of the contributions from those weak bands.

3) For short peptides reconstituted in lipid membranes, when the sample is dried, the absorbance bands usually have a sharp shape, especially for $\beta$-sheet band. Thus for a complex band consisting of the mixture of sharp $\alpha$-helix ($\sim$1650 cm$^{-1}$ and $\beta$-sheet ($\sim$1627 cm$^{-1}$) bands, the baseline determination method can be used to distinguish the $\alpha$-helix band from $\beta$-sheet structure. If the $\alpha$-helix band is the major band, the intensity of the $\alpha$-helix band can be read directly and a baseline will be set for the $\beta$-sheet band. If the $\beta$-sheet band is the major band, the intensity of the $\beta$-sheet band can be read directly and a baseline will be set for the $\alpha$-helix band.

Certain errors may be introduced by using the baseline determination method except for an isolated band. However, since certain errors will be also introduced in curve-fitting method, baseline determination method can be used as an alternative for the limited cases discussed above. The determination of dichroic ratio R with the baseline determination method should be regarded as a qualitative method rather than a quantitative method considering the possible errors. Same method to determine the dichroic ratio R should be used for all samples in a related work.
For a featureless broad complex amide I band from a large protein, the baseline
determination method cannot be used and the curve-fitting method will be much less
accurate too.

A.1.3.6 Buffers and salts in sample solutions

When sample solutions were dried on crystal surface, buffer substances and salts
in sample solutions may influence the packing and orientation of the multilayer on the
crystal surface, possibly due to the formation micro-crystals by the buffer salts. It is
preferable to disperse membrane fragments in water only to enable the formation of
well-orientated sample membranes on the crystal surface.

However, if the pH of the sample solutions needs to be controlled, the total
buffer substances and salts used should not largely exceed the mass of the sample to be
analysed (Goormaghtigh et al., 1990). The buffer should contain no carboxylic acid,
such as acetate, or carbonate, which absorbs in the amide I band region.

For samples at neutral pH, a dilute buffer may be used. The mass of the buffer
should be much less than the mass of the sample (lipid, protein, peptide etc). After the
sample suspension is dried to form multilayers, the multilayer can be rehydrated by a
small amount of H₂O or D₂O, thus concentrating the buffer in the rehydrated sample.
Because the pH of the rehydrated sample cannot be checked, we assume that the pH of
the rehydrated sample is the same as before initial drying.

For acidic peptide samples at pH5, the acidic peptide itself serves as a buffer at
this pH (Rafalski et al., 1991). The pH of the sample can be adjusted with dilute HCl.
After the sample suspension is dried to form multilayer, the multilayer can be
rehydrated by dilute HCl in H₂O or DCl in D₂O at pH5 or pD5, and the sample is
assumed to be at pH5 (or pD5).