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Engineering of Light-Gated Artificial Ion Channels

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In memory of my parents
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1 AIM OF WORK

The engineering of artificial ion channels has received great attention to overcome the limitations of natural ion channels. The goal is to allow artificial ion channels to operate over a wider range of temperatures, solvents, voltages or pH than natural ion channels. Moreover, studies on artificial ion channels are needed to allow a more complete understanding of the complex processes that occurs in natural ion channels. Although a wide range of artificial models have successfully simulated the mechanism of natural ion channels, synthesising the features that control the sensitivity and reversibility of ion channels still remains a big problem that needs to be solved for these artificial systems. Artificial ion channels synthesised to date lack any externally controlled gating mechanism, which severely limits their applications. The switching mechanism of many artificial ion channels is a stochastic process determined by chance. The absence of a controlled gating mechanism has the consequence that the switch cannot be made to actuate at a certain moment.

Our present contribution is to solve this problem through the development of a rigid but reversible gating mechanism for artificial ion channels that is powered by light. In this thesis, the synthesis, photoisomerisation and basic patch clamp experiments are described. The gate could give artificial ion channels the capability to be used as molecular switches (nano-valves) in microfluidic systems, in biomimetic sensors (pharmaceutical, diagnostic applications) and various technical devices. The gate part of our system is based on light-responsive azo groups attached to the transmembrane channel formed by calix[4]resorcinarene cavitand. The key to the control mechanism is the conformational change between cis and trans isomers that is translated into movement of the gate. Channel activity will be suppressed and reversibly revived by UV-Vis irradiation. Information about ion channel activity and selectivity mechanism is provided by the patch clamp technique, commonly used for electrophysiological analysis.
2 ION CHANNELS

2.1 NATURAL ION CHANNELS

- What are natural ion channels?

Cells are surrounded by membranes that separate the interior of the cell from its environment and act as barriers for the ions. One way for ions and water to pass through such biological membranes from extracellular solution to intracellular solution and vice versa are ion channels. Ion channels are special protein complexes that create tunnels through the membrane to facilitate the diffusion of ions, water or small organic molecules (e.g., porine in Figure 1).

![Image of ion channel](image_url)

**Figure 1**: Natural ion channel (porine trimer type in a cell membrane)

Natural ion channels consist of two functional units: the pore and the gate. The pore allows the ions to penetrate through the membrane, while the gate gives the mechanism for switching on and off the ion flux. The ions that pass through the channel are considered as an ion flux that control a multitude of body functions.
The gating mechanism is very important since the proper function of nerve and muscle tissue is dependent on the exchange of ions through ion channels at specific moments. During the gating process, the channel conformation and membrane permeability is successively changed and involves the obstruction or the liberating of the pore part of the ion channel. The open and closed states depend on signals coming to ion channels from either inside or outside the cell. Due to the gating mechanism of channels, the cell is able not only to facilitate the diffusion of ions across its membrane but also can check and manipulate the passage of ions. Many of these channels achieve the performance to have a rapid rate of change: they stay open for a few milliseconds or less before closing again. Thus they have an extremely fine level of control over what goes into and out of the cell. That the reaction takes place in milliseconds is an extremely important factor for survival in nature because of the amount of time it takes to respond to danger in the environment (e.g., the reflexive muscle movements responding to nerve stimuli). This can often mean the difference between survival and death.

- **What happens when ions go through the membrane?**

When the ions cross the cell membrane unaccompanied by their counterion (e.g., \( \text{Na}^+ \) without \( \text{Cl}^- \)) an electrical current in the range of picoamperes is generated. This current or charge creates a voltage difference across the cell membrane of a magnitude directly proportional to the rate of ion flux. The control of ion movements through the open channels is made via concentration and electrical forces. The balance in electrical charges across the cell membrane is known as the membrane potential. Every cell in our body is slightly more negative inside than outside.

- **Why are ion channels needed?**

Cell membranes create a very hydrophobic impermeable barrier to ions or charged molecules no matter how small they are. The movement of ions across the nerve cell membrane, which generate and propagate the excitation and electrical signals in nervous system, is possible via ion channels. From this point of view, ion channels are considered the key regulators of many fundamental processes in biology, from
fertilisation to nerve conduction, including communication between cells and the process by which stimuli such as light, sound, taste, heat or pH in areas of the body are converted into signals that can be sent to the brain.

- **Which ions go through ion channels?**

Potassium, sodium, chloride and calcium are the major inorganic ions that flow through ion channels. Cations like sodium and potassium have a crucial role in nerve impulse transmission. Not all the ions pass into or out the cell through the same type of ion channel. Based on their selectivity to ions, natural ion channels are divided into potassium channels\(^4,5\), sodium channels\(^6,7\), calcium channels\(^8,9\), etc. However, no channel is perfectly selective. For example, potassium channels are more permeable to potassium than other types of ions, while sodium channels largely select sodium ions over other ions.

- **What happens when ion channel functions are altered?**

When ion channel functions are damaged, the cellular integrity and functions are damaged and the outcome can be devastating. Dysfunction of ion channels is related to many common diseases, such as diabetes\(^10\), hypertension\(^11\), angina pectoris\(^12\), kidney stones\(^13\), epilepsy\(^14\) or other psychiatric diseases\(^15\). Epilepsy can result from defects in calcium channels\(^16,17\), while tendencies to kidney stones are known as chloride channel alteration\(^18\). Perturbations in potassium channels\(^19\) involve defects in the heartbeat and several types of inherited deafness. Moreover, potassium channel damage may have a huge effect on sleep in humans, changing the excitability of neurons\(^20\).

Sodium channel diseases\(^21\) appear as muscle spasms or high blood pressure (hypertension) while chloride channel malfunctions are indicated by a tendency to kidney stone formation.

On the other hand, many toxins can block ion channels\(^22\). Scorpions\(^23\), spiders or snakes\(^24\) use many of these toxic substances for defence or as a weapon. Most of these substances affect specific ion channels of the nerve cells.
• **Drugs targeting ion channels**

The ability to affect or modulate the ion channel activity allows researchers to develop many types of drugs and hormones. The effect of such drugs on cell function is exactly based on alteration of the ion transport across the membrane. One key approach for drug discovery is to screen for compounds that interact with ion channels in an efficient way. There is a vast array of drugs that influence ion channel function, including ones that treat epilepsy, heart conditions and migraines. Many neuromuscular blocking agents and hypnotic drugs work on the principle of binding the designed new drug agents to ion channels while the local anaesthetics drugs act by modulating or blocking sodium channels.

• **Types of natural ion channels. Natural gating mechanism**

According to their gating mechanism natural ion channels can be classified as: (i) ligand-gated (nAChR), (ii) voltage-gated, (iii) light-gated (rhodopsin), (iv) volume-regulated.

Ligand-gated channels can be opened or closed in response of binding to transmitter molecules. The gating is made by ligands that bind to a receptor in the channel. Receptors are basically sections of proteins (e.g., nAChR with pentameric structure, Figure 2) that allow very specific molecules to bind and initiate changes in the protein conformation.

A single ion channel has several receptors for the same type of ligand or even more for very different types that allow either the opening or closing of the channel. Since ligand-gated ion channels receive signals from the ligand, they are also considered “receptors” for ligands. Ligand-gated ion channels (e.g., nAChR) are responsible for the transfer of nerve impulses from nerve cell to muscle.
Voltage-gated ion channels\textsuperscript{30} can switch between open and closed positions in response to changes in the potential difference across the cell membrane. Opening occurs when the membrane becomes more depolarised, while closing happens when the membrane becomes repolarised. The cycle of depolarisation and repolarisation is extremely rapid; it takes only about 2 milliseconds and determines the speed of the fast gating process. Since this type of ion channel can be opened and closed quickly, the richest source of voltage-gated channels is founded in the nervous system.

Light-gated channels\textsuperscript{31} can switch between open and closed positions under different light conditions through a complex biochemical cascade whereas volume-regulated ion channels...
channels become active according to how much tension is placed on the cell membrane due to swelling or shrinking.

- **Gramicidin A, the most studied natural ion channel**

The gramicidin A is a helical polypeptide composed of 15 neutral amino acids. Gramicidin A acts like an antibiotic against gram-positive bacteria. The ion channel conducting pathway across the membrane is built on the dimerisation principle (Figure 3). One molecule of Gramicidin A constitutes a “half channel” element moving around in a particular layer of the lipid bilayer. The ion channel pore forms by chance, when two molecules meet and form a dimer stochastically. The arranged head-to-head mode of the dimer is possible because the N-end of the gramicidin A molecule in the center of the membrane is stabilized by hydrogen bonds. Once the dimer is formed, the ion channel is considered to be in its open state and certain ions can pass through. The closed state appears upon dissociation of the dimer and no ions can pass through.

![Figure 3: Gramicidin A channel formation mechanism](image)

A: ion channel (tail-to-tail associated dimer);
B: no ion channel formation (monomers)
Ion channels

Gramicidin A channel is cation selective\textsuperscript{38}, although gramicidin A itself is extremely hydrophobic and contains no polar amino acids. Cation selectivity results from differences in ion transfer free energies from bulk water to the channel interior (difference between two large energy terms: the free energy of hydration and the free energy of interaction of an ion with the ion channel). Anions bind water of hydration more strongly. The anion stabilization free energy within the channel is greater than the cation stabilization energy\textsuperscript{39}. Conductance measurements have shown that the gramicidin A channel is permeable to monovalent cations and impermeable to anions and divalent cations. On the other hand, calculations indicate that anions would be quite stable inside the channel but the energy required for passing the channel entrance creates a kinetic barrier to anion transport\textsuperscript{40}. Simulation studies of gramicidin A\textsuperscript{41} show that 87\% of overall channel resistance to water permeation comes from the energetic cost for a bulk water to enter the cavity of the channel. Divalent ions, although they have appropriate radii, are impermeant because their hydration shells exchange too slowly. In fact, divalent ions act as channel blockers (binding near the channel mouth and occlusion of the channel)\textsuperscript{42}.

Gramicidin A channels have been used to design biosensors for the determination of proteins, viruses, antibodies, DNA, electrolytes and drugs\textsuperscript{42}. Based on the channel mechanism of gramicidin A, the construction of nanomachine ion-channel switches that are only 1.5 nm in size was reported\textsuperscript{43,44}. The basis of the device is an artificial membrane packed with gramicidin A. Ion channels are formed in the membrane by two gramicidin molecules, one in the lower layer of the membrane attached to a gold electrode and one in the upper layer tethered via covalent bonds to biological receptors such as antibodies or nucleotides. The binding of the target molecule splits the gramicidin A dimers to non-conducting monomers. The device converts a biochemical signal into an electrical signal and is capable of detecting pico-molar concentrations of proteins.
**Hybrid ion channel**

A few specialized animal cell types (such as rods and cones of the retina) have naturally photoreceptive proteins (e.g., rhodopsin). These receptors indirectly signal to ion channels through a complex biochemical cascade but none is known to be directly sensitive to light. The natural gating mechanism is operated by photo-excitation of the chromophore group (e.g., rhodopsin), which leads to a trans-cis transformation (open-closed positions) in only two hundred femtoseconds\(^{45}\). Because rhodopsin signals through a complex biochemical cascade, several proteins need to be present for rhodopsin to allow nonphotosensitive cells to generate even a rudimentary light response\(^ {46}\). Moreover, outside their environment the light-gated natural ion channels do not survive prolonged irradiation\(^ {47}\). It is this extraordinary light sensitivity together with the difficulty of isolating and purifying such natural channels that makes these molecular switches unsuitable for use in a biosensor.

**Figure 4**: SPARK hybrid ion channel (artificial light gate formed by azobenzene developed on natural Shaker channel\(^ {48}\)). A: closed channel state at 500 nm, B: open channel state at 380 nm
Ion channels

The development of chemical tools for optical stimulation or inhibition of proteins has a particular relevance for the nervous system, where precise, noninvasive control is an experimental and medical necessity. In the past few years, many efforts have been undertaken to directly activate or inhibit ion channels by light and thus to control the cellular functions. Kramer and coworkers reported a successful hybrid ion channel that can be opened and closed directly via light\textsuperscript{46}. The supplementary artificial light-operated gate is based on a photoswitch molecule attached to the voltage-gated natural Shaker ion channel. Shaker channels are voltage-gated and they are found in neurons. However, neurons have natural ion channels that are directly controlled by voltage, ligands and temperature but not by light.

The hybrid ion channel (Figure 4) called SPARK (synthetic photoisomerizable azobenzene-regulated K\textsuperscript{+} channel) has the advantage that it exhibits double gating mechanisms that confer both light and voltage sensitivity to modified Shaker channels\textsuperscript{48}, dramatically improving the control. Due to the light-gating artificial mechanism, this hybrid approach allows neurons to restore the light-regulated activity in healthy retinal neurons after degeneration of native photoreceptors (rods and cones). The hybrid approach can be opened and closed rapidly (<100 ms).

![Figure 5: UV-Vis spectrum of SPARK hybrid ion channel (3 minutes time irradiation exposure for both transformation: trans-cis isomerisation respectively cis-trans isomerisation)\textsuperscript{48}](image)
For opening the channel, 380 nm light is optimal while 500 nm light is optimal for accelerating its closure (Figure 5). However, when this compound is kept in the dark for several minutes, the SPARK channel spontaneously reverts to its blocked state (thermally relaxes from the cis to trans configuration).

The SPARK hybrid channel with the double gating control mechanism was tested successfully only within the biological cells\textsuperscript{48}. Outside the natural environment, the ion channel is quite sensitive to pH changes, which may fully inhibit its functionality\textsuperscript{49}. Changes in the electrochemical potential of membrane may even cause irreversible gating of the channel\textsuperscript{50}. All these impairments were observed even for the most stable natural ion channels if they are used in an artificial environment. The problem of radically diminished performance of natural ion channels in a wider range of temperature, solvents, voltages or pH was tackled through the development of artificial ion channels that mimic the natural ion channels.

2.2 ARTIFICIAL ION CHANNELS

Many research efforts have been directed to the engineering of artificial ion channels in order to create systems with higher stability, better handling and more applications as biosensors. Natural ion channels features like sensitivity and selectivity bring with their varieties and complexity many models into the research work. Natural ion channels have the benefit of millions of years of evolution to optimise their properties. These capabilities caused chemists to look for possibilities and ways to design the achievements of the natural ion channels by creating biomimetic systems. Moreover, a variety of designs that mimic the ion transport in biological systems is needed for understanding the complex processes of ion transport through the membrane.

To create stable artificial ion channels, three strategies have been observed. One strategy tries to introduce nanopores into thin metallic\textsuperscript{51} or polymer films\textsuperscript{52}. Chemical modifications (hydrophobicity, polarity) of the lining of the channel wall can hardly be
achieved for such pores. The second strategy is directed towards modification of simple natural ion channels. The third strategy aims at the synthesis of novel molecules that mimic the required features of the natural ion channels without duplicating their fragile structure. The focus of our work is on the third strategy. This provides more freedom for modifications in every part of the synthesized molecule and specific aspects of the channel function can be assessed.

The synthesis of novel molecules that mimic the required features of the natural ion channels is approached in two different ways. The molecules are designed either with a peptidic macrocycle structure or with a non-peptidic structure. Since the peptides used as channel models are compositionally close to natural channel proteins, the non-peptidic models are particularly attractive because they provide new approaches to designed robust artificial ion channels that can be modified according to the class of analytes which should be detected. Moreover, the non-peptidic artificial channels are an important step because they challenge the theory that such models must be constructed from peptides. A large number of non-peptidic structures like calix[4]resorcinarenes, amphiphilic ion pairs, cyclodextrin, crown ethers and cholic acid derivatives have already been proved as ion channels. Both types, the peptidic and non-peptidic artificial ion channels have been designed both with hydrophilic and hydrophobic interior pore walls.

2.2.1 Artificial channels with a hydrophilic interior wall

The capability to control and manipulate different types of structures with nanometer dimensions holds immense potential for the development of artificial ion channels. The best known and studied artificial channels are designed with a polar interior wall of the pore (which supports the ion translocation from solution to the channel) surrounded by a non-polar exterior wall (which permits the integration into a lipid bilayer). The main strategies for the engineering of artificial ion channels with hydrophilic interior wall are focused on different artificial peptidic research.
Considerable effort has been directed towards artificial peptides with cationic beta-helical conformation (Figure 6). Such types of peptides were reported to form synthetic ion channels within lipid bilayer membranes\textsuperscript{59}. Several membrane active helices containing beta-aminoacids have been reported over the years\textsuperscript{60,61}. 

**Figure 6:** Different types of cationic beta-peptide backbone in helical conformation with alternating amino acids residues that exhibit ion channel activity\textsuperscript{59}
Figure 7: The chemical structure of a 24-membered macrocyclic molecule composed of alternating D- and L-amino acids, cyclo[Gln-(D-Leu-Trp)₄-D-Leu] (left) and a self-assembled tubular structure spanned across the bilayer lipid membrane (right).²⁶²

Ghadiri and coworkers²⁶² have applied a specific peptidic strategy for the new class of artificial ion channels. They employed an eight-residue 24-membered macrocyclic peptide with the sequence: cyclo[-(Trp-D-Leu)₃-Gln-D-Leu] (Figure 7). The units stack in an antiparallel fashion with intermolecular hydrogen bonding to produce a continuous β-sheet structure. In order to make the self-assembled molecule membrane-spanning, about eight or ten units are required to be stacked. The alternating D- and L-amino acid structure arrangements may find an analogy with gramicidin A channel. Subsequently the ion channel activity was tested by the patch clamp measuring technique.
Figure 8: α-helix peptide with benzo-type crown ethers (left) and the proposed functional state of the protein in a lipid membrane where the positions of the crown ethers are marked by circles (right)\textsuperscript{63}

Another strategy was proposed by Voyer and coworkers, who used crown ether fragments and a stable α-helix peptide to construct a new architecture on the basis of artificial ion channels. They have employed a rigid amino acid peptide helix (Figure 8) composed of L-alanines and six benzo-type crown ethers\textsuperscript{63,64} (R in Figure 8). Circular dichroism and fluorescence spectroscopy were used to demonstrate that this peptide forms an α-helix conformation and the arrangement of the crown ethers is located on the same side of the helix. The six crown ether units form a transmembrane ion channel within a lipid membrane (Figure 8). The cation transport capability of this type of peptide was reported to be comparable to that of gramicidin A.

2.2.2 Artificial channels with a hydrophobic interior wall

Despite most of the artificial ion channels being designed with hydrophilic interior walls, efforts have been made to create artificial ion channels with hydrophobic interior walls.
Different types of artificial ion channels, peptidic and also non-peptidic, were developed with a hydrophobic pore. The pore is mostly formed by acyl (Figure 9) or alkyl chains.

Figure 9: Cyclic peptidic compound with acyl chains (left) and the ion channel formation based on dimerisation of the cyclic peptidic compound (right).

A series of novel peptidic artificial channels based on cyclic peptides with long hydrophobic acyl chains provided channel function with cation selectivity\textsuperscript{65,66}. The non-polar pores formed by acyl chains exhibit van der Waals interaction with intrapore water molecules. This indicates that even a hydrophobic pore can be water-filled and ions move much faster through the central part of this hydrophobic pore than through the peptide ring region\textsuperscript{67}.

Furthermore, the hydrophobic pore model is supported not only by peptidic artificial compounds but also by certain non-peptidic molecules like calix[4]resorcinarenes\textsuperscript{53} or amphiphilic ion pairs\textsuperscript{54}. The novel calix[4]resorcinarene cavitand class is one of the fundamental non-peptidic models for artificial ion channels with hydrophobic pore walls\textsuperscript{68}. 
Calixresorcinarene cavitands (Figure 10) and resorcinol-derived calixarenes cavitands are subclasses of macrocyclic calixarenes. Normally, calixresorcinarenes are obtained by an acid-catalysed condensation reaction between resorcinol (C₆H₆O₂) and an aliphatic or aromatic aldehyde (e.g., C₁₂H₂₄O). Bridging of the phenolic hydroxyl groups in resorcinarene forms cavitands. Compared to the parent resorcinarenes, cavitands are extremely rigid molecules. They adopt a crown-like conformation with C₄ᵥ symmetry in the solid state and only slightly deviate from this structure in solution⁶⁹.

The name cavitand was given for the class of synthetic organic compounds with a concave cavity formed by aromatic rings, which is sufficiently large to accommodate...
Ion channels

other molecules or ions\textsuperscript{70}. The binding property of cavitands has been intensively studied in the solid state\textsuperscript{71}, gase phase\textsuperscript{72} and organic solvents\textsuperscript{73,74}. The cavitand class is particularly attractive because the rims of the cavity can be varied by different alkyl substituents (R in Figure 10). The size of the cavity can be also varied using different numbers of aromatic rings bridged by different types of groups. Such groups change the shape of the cavity and the acceptance of the cavitands\textsuperscript{75} for solutes. Moreover different functional groups (R’ in Figure 10) can be introduced to act as catalysts\textsuperscript{76}.
In the case of artificial ion channel formation, the rigid cavity of such compounds provides the channel entrance and a permanent free volume for ions.
The four long alkyl chains (R in Figure 10) reach into the bilayer and form the hydrophobic channel pore wall. Such supramolecular structures capture great interest since (i) they are simple enough to be understood and modified, (ii) they form single channels with one conductance level (as in natural channels), (iii) they can recognize monovalent cations, and (iv) they display selectivity for K\textsuperscript{+} ions\textsuperscript{77}.

\subsection{2.2.3 Selectivity mechanism of artificial ion channels}

The selectivity feature, one of the main properties of the ion channels, is caused by the energetic and geometric complementarity of the ions and the ion channel pores. The selectivity is closely dependent on the pore size of ion channels and the size and charge of ions that go through the channel, on the electric field of aromatic cage, on the energy of ion dehydration, on the electrostatic interaction between the ions and the pore of ion channel, on the equilibrium between host-guest complexation and decomplexation (host-pore, quest-ion) and on the membrane fluidity. It is very important that the host-guest complex is not stable in order to avoid irreversible complexation. Ions must leave the complex in order to go through the channel and not block the entrance of the pore.
In the case of artificial ion channels based on calix[4]resorcinarene cavitands, the ion selectivity is determined by a selectivity filter. This selectivity filter consists of the aromatic cage which provides a weak electric field that allows ion dehydration\textsuperscript{77}. The
π electrons of the aryl groups contribute to the lowering of the potential barrier for the passage of ions. Cations bind near the π face of an aromatic system through a strong, non-covalent force called cation-π interaction. This interaction can be considered as an electrostatic attraction between a positive charge and the quadrupole moment of the aromatic rings. Cation-π interaction is often used as selectivity filter in natural ion channels\textsuperscript{78}. Nature places aromatic residues (aromatics in general or aromatic amino acids) in the active regions of proteins to introduce a great selectivity filter and create a biological recognition site\textsuperscript{79}. Cations from simple ions like Li\textsuperscript{+} to more complex organic structures like acetylcholine (ACh)\textsuperscript{+} and N(CH\textsubscript{3})\textsubscript{4}\textsuperscript{+} are strongly attracted to the π face of benzene and other aromatic structures.

A binding side made up from aromatic rings binds cations by competing with the highly favourable solvation of an ion provided by an aqueous medium. Benzene is generally considered to be a non-polar molecule because it does not have a permanent dipole moment. It does, however, have a quadrupole moment that is quite substantial in magnitude\textsuperscript{80}. The quadrupole moment can be thought to be made up of two dipole moments aligned in such a way that there is no net dipole. This can be achieved by the tail-to-tail arrangement, or by placing two dipoles side by side, with one facing “north” and the other “south”. Thus, the benzene ring has a permanent, non-spherical charge distribution that is expected to interact with appropriately positioned charges through electrostatic forces (Figure 11).

In a polar molecule, such as water, there is a greater negative charge on the end with the oxygen atom, because oxygen pulls electrons more strongly than hydrogen atoms. Technically this molecule has a “net dipole moment”. In case of benzene, no single carbon atom is more negatively charged than the other carbon atoms. In this sense, benzene is nonpolar and there is no net dipole moment. However, the electrons in benzene are not evenly distributed throughout the molecule.
The carbon atoms, which are slightly more electronegative than the hydrogen atoms, pull the electrons closer to themselves. A higher negative charge (Figure 11 - blue colour) will be located in the center of the molecule and a more positive charge (Figure 11 - red colour) indicating lower electron density, located near the hydrogen. This quadrupole moment can be measured experimentally\textsuperscript{82,83} or can be calculated using quantum mechanical methods\textsuperscript{84}.

A unique feature of aromatic moieties is their combination of two properties: ion binding (e.g., interactions of resorcinarene with cationic species\textsuperscript{85,86}) and hydrophobicity. It was demonstrated that the cation-\(\pi\) interactions are responsible for establishing ion selectivity in K\(^+\) channels\textsuperscript{87,88}. Not only biological systems but also peptidic and non-peptidic artificial ion channels with a selectivity filter based on cation-\(\pi\) interaction have been reported\textsuperscript{89,90}. Such artificial ion channels are able to pull a cation out of water into a hydrophobic, nonpolar environment. The cation-\(\pi\) interaction competes with full aqua solvation energy of ions. Due to cation-\(\pi\) interactions, nonpolar environments composed of hydrocarbon units are energetically favourable to bind polar cationic substrates strongly and selectively\textsuperscript{65,66}. The hydrogen bonds, the all-important intermolecular interaction, are in competition with the cation-\(\pi\) interactions\textsuperscript{91}. 

\textbf{Figure 11:} Electrostatic potential images of benzene (Blue indicates more negative charge; red more positive charge)\textsuperscript{81}
### 2.2.4 Engineering of light gated artificial ion channels

The photosensitive azobenzene molecule is one of the best candidates for such systems because the photoisomerisation of azobenzene is one of the simpler and faster photochromic reaction. Z-E and E-Z isomerisations are extremely fast processes. Factors such as solvent viscosity, polarity or hydrogen bonding\(^{92,93}\) can influence the isomerizations, especially in the case of push-pull compounds\(^{94}\). It was reported that the rate of regeneration of E-azobenzene is enhanced by electron-donating substituents and by polar reaction media\(^ {95}\). Electron-withdrawing substituents generally suppress the rate of regeneration of E isomer in the dark (decrease of rate constant of “dark isomerisation”).

Since the ground energy state of the E-isomer is lower in energy than that of the Z-isomer, any Z species created can return to the E form either through a photochemical and (or) a thermal mechanism. For most common azobenzenes, the two photochemical conversions (from E to Z state and from Z to E state) occur on a picosecond time scale, whereas the thermal relaxation from Z to E state is much slower (in the order of hours)\(^ {96}\).

Azo groups possess lone pair electrons on both nitrogen atoms; hence they can isomerise via two distinct mechanisms because the presence of doublets indicates both n-π* and π-π* transitions. In case of the n-π* transition, the isomerisation process is induced by the electronic excitation of an electron from the highest occupied non-bonded orbital (n). The π-π* transition occurs between the highest occupied bonded orbital π (HOMO) and the lowest unoccupied anti-bonding orbital π* (LUMO).

It has been proposed that visible excitation (n-π* transition) of azobenzene results in Z-E isomerisation by an in-plane inversion mechanism centred on one of the azo-group nitrogen atoms, with inversion of the nitrogen as well. The “lateral shift mechanism” (inversion of the nitrogen doublet) is proposed to occur through a planar transition state with conversion of the double bond in the azo group to a single bond. The transition state should have sp-hybridised geometry at the N-atom (rehybridization mechanism) and the π-system should be hardly involved\(^ {97,98}\).
UV excitation of azobenzene (\(\pi-\pi^*\) transition) has been proposed to result in E-Z isomerisation by a large-scale rotational motion around the N=N bond (rotation mechanism).

Subsequent theoretical calculations indicate that the photoisomerisation of E-Z mechanism of the azobenzene can also proceed via an inversion mechanism of one or both of the nitrogen atoms even in the case of the \(\pi-\pi^*\) excitation\textsuperscript{97}. This problem is still under discussion but, since both mechanisms are possible in the photochemical E-Z isomerisation process, the free electron doublet inversion mechanism often competes with the simple rotation around the N=N bond.

**Figure 12:** Possible mechanisms of azobenzene isomerisation via rotation (\(\vartheta\)) and inversion (\(\theta\))

The arrows on the molecular structure illustrate that rotation (\(\vartheta\)) occurs by twisting of a phenyl group around the N-N bond, whereas inversion (\(\theta\)) occurs by in-plane motion of a phenyl group through a linear N-N phenyl geometry\textsuperscript{99} (Figure 12).

The Z state of azobenzene is characterised by two bands in the absorption spectrum (one intense band in UV range and a very weak band in VIS range) while the main feature of the E isomer is an intense band in UV range well separated from both Z bands\textsuperscript{100,101}.

The difference in intensity in the absorption spectrum is due to the selection rules: in the planar C\(_{2h}\) and C\(_{2v}\) symmetries, the n-\(\pi^*\) transition is “forbidden” for the trans isomer (E), but allowed for the cis isomer (Z). Under this aspect the intensity of the band in the
spectrum of the trans form is very high. This has been attributed to non-planar distortions of the molecule and to vibrational coupling. The “forbidden” transitions are not actually forbidden but only highly improbable.

In case of $\pi-\pi^*$ transition, the molar absorptivities, $\varepsilon$, are very large ($\varepsilon > 10.000$). In case of $n-\pi^*$ transition and very small if the absorption is weak ($\varepsilon = 10$ to $100$). The magnitude of $\varepsilon$ reflects the probability that light of a given wavelength will be absorbed when it strikes the chromophore. The general equation is as follows:

$$\varepsilon = 0.87 \cdot 10^{20} P a,$$

where $P$ is the transition probability (0 to 1) and $a$ is the area of the chromophore in cm$^2$. The factors that influence the transition probabilities are complex. One important factor is the overlap of the orbitals involved in the electronic excitation. In case of azobenzene isomerisation, the $n-\pi^*$ transition is lower in energy than the $\pi-\pi^*$ transition, but $\varepsilon$ of the former is far smaller than that of the latter. This situation is caused by the spatial distribution of these orbitals. The $n$-orbitals do not overlap well with the $\pi^*$ orbitals, so the probability of excitation is very small. The $\pi-\pi^*$ transition, on the other hand, involves orbitals that have significant overlap and the probability is very close to 1.$^{102}$
3 LIPOID BILAYERS

3.1 NATURAL BILAYERS

Natural ion channels do not work at all if they are not integrated into a membrane. Biological membranes are bilayers of lipids that are organized to form a long non-polar hydrophobic core surrounded by two hydrophilic interfacial layers. The hydrophobic organization is stabilized by hydrophobic forces while the hydrophilic layers are stabilized by van der Waals forces. The most polar external parts of a membrane interact with the external aqueous compartment of a cell. The membrane lipids and proteins can diffuse laterally, implying that the membrane is, in fact, a two-dimensional fluid mixture. The elastic and compositional (lateral diffusion) degrees of freedom of lipid membranes play a crucial role in their interactions with proteins which can induce local changes in the curvature, thickness and composition of its lipid surroundings.

Biological membranes act in a similar way as capacitors. Biological membranes separate solutions in two compartments by the very short distance, non-conducting, hydrophobic core of the membrane (approx. 3 nm). Charge separation across the membrane leads to an electric field across the membrane. This electric field gives rise to the measured membrane potential.

The first work on the self-assembly of the lipid membrane in vitro was carried out by Mueller and Rubin and their co workers. They showed that a lipid membrane formed from brain extracts was self-sealing to puncture with the following characteristics: capacitance ($C_m$) at about 1 $\mu$F cm$^{-2}$, resistance ($R_m$) greater than $10^8$ ohm cm$^{-2}$ and dielectric breakdown potential ($V_b$) at about 300 KV cm$^{-1}$. Furthermore, upon modification with suitable proteins, this membrane became excitable displaying characteristic features similar to those of the action potential of the nerve membrane.
3.2 ARTIFICIAL BILAYERS

Artificial bilayers are used as models for biological membranes. They are two-dimensional fluid mixtures that have many similarities to biomembranes, for example, their resistance and capacity, thickness and permeation by water are all similar\(^{108}\) to the corresponding parameters of biological membranes.

![Figure 13: The phospholipid bilayer with hydrophobic and hydrophilic regions](image)

Artificial bilayers are designed in two complementary subunits: a hydrophilic moiety and a hydrophobic moiety with a low dielectric barrier for hydrophilic and charged molecules.

The first approach for construction of an artificial planar lipid bilayer involved painting a “forming solution” over a hole in a Teflon septum that separate two aqueous compartments of a chamber, followed by the spontaneously thinning to a lipid bilayer.
Lipid bilayers

The “forming solution” consists of a surface-active lipid “dissolved” in a nonpolar liquid.

The bilayer forms spontaneously by painting a lipid solution across a small (1-2 mm) aperture in a hydrophobic polymer separating two aqueous phases. The lipid solution consists of a surface active lipid dispersed in a non-polar liquid, like n-decane or squalene.

Figure 14: Schematic process of planar lipid bilayer formation from lipids (dispersed in alkane solvents) applied across an aperture in a nonpolar material (such as polychlorotrifluoroethylene). 1-thick film; 2-bilayer, 3-annulus

The painted bilayer is formed from dispersions of phospholipids in such non-polar solvents. As the lipid solution drains to the border of the aperture, a film is formed in the central part of the aperture (Figure 14). This film gets thinner and finally become a bilayer. This happens under the influence of several driving forces. The major one in the early stages is the Plateau-Gibbs border suction, which arises from the curvature of the annulus (Plateau-Gibbs border). The curvature is necessary for the contact angle that the border must make with the pore wall of polymer and the film. Wherever there is a curved interface at mechanical equilibrium, there must be a hydrostatic pressure across it. The pressure is always greater on the concave side of the surface so that the
Lipid bilayers

pressure in the border must be less than in the flat region. Therefore, bulk solution will flow from the flat film into the border, causing the film thinning. When the film achieves a thickness of a few nm, a second driving force becomes apparent. It is the London-van der Waals attraction between the aqueous phases separated by the thin film. This force leads to the final thinning and is the major force attempting to thin the membrane at the equilibrium bilayer thickness. The bilayer film usually appears first at the center of the film and propagates rapidly over the surface and is surrounded by an annulus of the bulk solution. The free-standing bilayer and the annulus are interconnected and reside in equilibrium.

Because the mass of the annulus is a million times bigger than that of the bilayer, the chemical potential of the components in the bilayer tends to be the same as that of the annulus. To control the annulus means to control the bilayer. The presence of the lipid bilayer can be easily checked by measuring the electrical resistance, which drops from $10^8 \ \Omega$ to $1 \ \Omega$ when the membrane collapses. The reciprocal of electrical resistance is called electrical conductance measured in siemens (S, equal to $1/\Omega$), which measure the ease of flow of current between two points.

In contrast to the formation of the painting technique, the generation of solvent-free planar bilayers from monolayers is based on the fusion of two monolayers. Many groups until now have used this method with different diameters of the hole in the teflon septum (e.g., 0.5 cm).

Later, lipid bilayers were constructed using Langmuir-Blodgett (L-B) and self-assembly monolayer techniques on the interface of air and water.

Another methodology that has been used since 1977 involved the formation of a supported lipid bilayer on solid and gel substrates. Later, supported lipid bilayers were formed on metallic wires, on conducting SnO$_2$ glass, gold substrate, and on microchips. These self-assembled supported lipid bilayers can overcome the long-term stability problem of conventional lipid bilayers. But in the case of ion channel formation through supported lipid bilayers there are a few disadvantages; these include relatively poor membrane fluidity and the absence of a sufficiently aqueous environment in the substrate phase.
Lipid bilayers

Many attempts have been made to stabilize the extremely delicate lipid bilayer structure and simultaneously to keep the activity of the ion channel. Based on such consideration, considerable effort has been focused on the construction of lipid bilayers in porous polymer filters\textsuperscript{125,126} as well as on the reconstitution of ion channels either in lipid-treated filter\textsuperscript{127} or using gel-protected membrane\textsuperscript{128,129}. The stability of the lipid bilayers is improved with respect to both chemical and mechanical disturbance.

The selection of a solvent that will yield a controlled environment for ion channels in the bilayer received much attention. The annulus controls the formation of the lipid bilayer and exhibits a complex phase behaviour. For this reason, the mixture should not be a simple solution of lipid in organic solvent. The lipid is present in the solvent as hydrated aggregates (micelles), which tend towards equilibrium with the bulk aqueous phase. In the very first experiments of lipid bilayer research, standard lipid extraction solvents such as chloroform or methanol were used, but for the formation of the lipid bilayer proved to be difficult because of the time-dependent changes in composition and phase behaviour in the presence of excess water. Later, the most common nonpolar solvents used were alkanes or other long-chain hydrocarbons such as squalene. Also in the case of artificial ion channels, integration in the lipid bilayer was achieved using the painting method with hydrocarbon-containing solvents, such as octane, hexadecane, n-decane or squalene\textsuperscript{130}.

The lipid solution was also mixed with cholesterol. On one hand, the cholesterol increases the fluidity of the lipid bilayer when the temperature is low and on the other hand decreases the fluidity in high temperature by restricting rapid movement of the hydrocarbon chain of lipid molecules. Furthermore, the cholesterol also forces the polar head groups of lipid molecules further apart to increase access to the bilayer interior for the molecules at the membrane surface. Moreover it helps to seal the non-polar part of bilayer against the smaller ions and molecules, which may accidentally penetrate the bilayer.

Cholesterol has a concentration-dependent effect on the lipid bilayer properties and it is able to control its fluidity and permeability.
The most used bilayer for the integration of ion channels is the phospholipid bilayer (Figure 13). Such phospholipid bilayers are not permeable to cations ($\text{K}^+$, $\text{Na}^+$, $\text{Ca}^{2+}$), anions ($\text{Cl}^-$, $\text{HCO}_3^-$) or small hydrophilic molecules like glucose. Charged molecules or atoms could not penetrate this barrier without ion channels. The bilayer barrier to free diffusion of ions was first considered by Parsegian who treat the hydrocarbon phase as a solvent in which an ion might dissolve. The energy required was calculated in terms of the Born energy necessary to move a monovalent cation from water (dielectric constant = 80) into the hydrocarbon phase of a lipid bilayer (dielectric constant = 2). Parsegian showed that the monovalent cation needs a substantial energy to move from water into the hydrocarbon phase of a lipid bilayer. This substantial energy explains why a lipid bilayer about 3 nm thick forms an effective barrier to ionic diffusion. The barrier can be overcome by providing a hydrated channel. Parsegian showed that a hydrated 0.5 nm diameter pore into lipid bilayer reduces the Born energy barrier from 0.966 J/mol to 0.162 J/mol, allowing specific ions to cross the lipid bilayer. Gramicidin A channels are classical examples of such pores.
4 INSTRUMENTS AND CHEMICALS. EXPERIMENTAL DETAILS

4.1 INSTRUMENTS

4.1.1 Patch clamp system

Key information about channel gating and ion transport is provided by the patch clamp technique, commonly used for electrophysiological analysis. The patch clamp technique was developed by Neher and Sakmann in 1976 for the study of very small ion currents through single ion channels in cell membranes\textsuperscript{134}. The patch clamp technique shows that an individual ion channel fluctuates between states that are closed and open to the flow of ions.

The patch clamp set-up is build from optical, mechanical and electrical components.

- **Patch Clamp Optical Components**

The optical components consist of a microscope (for membrane visualization) placed on a vibration isolation table within a Faraday cage and a video camera. For our measurements we used an inverted microscope IX 70 (Olympus, Hamburg, Germany) that allows phase contrast and differential interface contrast (DIC). The objective lenses are CPL 10x PH and LCPL 60 PH.

In order to monitor the changes in the membrane under investigations we used a video camera (CD COLOR VIDEO CAMERA DXC-107P, Sony Corporation: Hoofdorp, Germany). This camera was attached to one of the lateral optical outputs of the microscope. A video monitor PVM-1440QM was used as display.
• **Patch Clamp Mechanical Components**

The mechanical components used for the patch clamp set-up consists of: a vibration isolation table, Faraday cage, micromanipulator, pipette holder and sample chamber. The vibration isolation table used is an air-suspension table (Science Products: Hofheim, Germany) that damps out the microscopic movements and vibrations that are present at different degrees in the building. Most low-end air suspension tables available from optical companies will reduce vibrations beyond a few Hertz in frequency, which is usually sufficient for the purpose of patch clamp measurements.

To shield the sensitive patch clamp measurements from electrical noise, the patch clamp setup is protected by a Faraday cage.

In order to have a sensitive control of the patch pipettes a motorized 5171 Eppendorf micromanipulator (Eppendorf Netheler-Hinz, Hamburg, Germany) was used. The 5171 micromanipulator is controlled via a joystick, installed on a separate desk outside the Faraday cage.

A patch pipette holder used for adjustment of patch pipettes was purchased from Heka Electronics (Lambrecht, Germany).

To obtain a negative pressure in the patch clamp system, a manual piston pump (CELL TRAM AIR from Eppendorf Netheler, Hamburg, Germany) was used.

A special type of sample chamber for patch clamp measurements was developed in our institute in order to study the reconstitution of ion channels into the artificial lipid bilayer formed into a porous polymer matrix. The measuring chamber was made of poly(tetrafluorethylene) (PTFE) with a small pore diameter of 0.6 mm. A special type of polymer film, poly(ethylene terephthalate) (PET) polymeric micropores with pore diameter: 10µm, thickness: 16 µm, 15% porosity was used as the matrix for ion channel formation. The polymer was attached onto the pore of the PTEF chamber by using a strong silicone adhesive (Dow Corning 3140, Dow Corning Corporation, Midland, USA).
• **Patch Clamp Electrical Components**

Two electrical components were used for the patch clamp set-up: an amplifier and an oscilloscope. The amplifier used for our system is EPC-9, a completely computer controlled amplifier from Heka Electronics (Lambrecht, Germany). The EPC-9 has a capacitive feedback and records the channel activity, combining the hardware of the amplifier with the operating and data acquisition software.

• **Patch Clamp Data Acquisition and Analysis**

Calibration of the EPC-9 amplifier was performed by the program E9 SCREEN v8.11. Data acquisition of the patch clamp measurements was executed with the program PULSES v8.11 and PULSES TOOLS v8.11. Single-channel analysis of the recorded patch clamp data was carried out with programs TAC v2.6.0 and TAC FIT v2.6.0. The programs E9 SCREEN, PULSES, PULSES FIT and PULSES TOOLS are from Heka Electronics (Lambrecht, Germany) The program TAC and TAC FIT are from Scalar Instrument Inc. Seatle, USA. The PULSE package is the main acquisition program, while the PULSE FIT allows the user to analyse the PULSE DATA.

• **Preparation of Patch Clamp Pipette**

Patch Pipette

The patch pipettes used for our experiments were made from borosilicate glass capillaries, type GB100-10 from Science Products (Hofheim, Germany). These patch pipettes have a length of 100 mm, an inside diameter of 0.58 mm, an outside diameter of 1.00 mm and a wall thickness of 0.21 mm. Borosilicate glass was chosen for the patch clamp experiments because it has a favourable melting point range (700 - 850 °C). This made the pulling of the glass by using the pipette puller more efficient and hence allowed patch pipettes of defined thickness in contrast to the soft soda glass (m.pt. < 700°C), hard aluminum silicate glasses (m.pt. > 900°C) or quartz glass (m.pt. ~ 1600°C).
Before clamping the patch pipette to the pipette holder, the pipette was filled with the electrolyte solution. This was followed by removal of any bubble by shaking the patch pipette. The pipette was filled from the rear with the help of a Microfil™ needle mounted on 5 mL syringe with a 0.02 µm particle filter (Nalga Company, Rochester, USA). The level of the electrolyte solution was set to the middle of the pipette so that it did not wet the inside of the pipette holder. This pipette is used as microelectrode in electrophysiological recordings.

**Pipette Puller**

The glass capillaries pulling took place in two steps using a horizontal microelectrode puller model P-97 made by Sutter Instrument (Novato, California, USA). The glass micropipettes are prepared by heating the center of a glass capillary tube until it becomes soft, and pulling the two ends apart such that the center of the tube tapers to a very fine point and finally breaks (resulting in two glass micropipettes each with a fine tip).

- **Preparation of the Cl⁻ (aq) | AgCl (s) | Ag (s) Electrodes**

Electrical connection of the chamber to the measurement equipment is done by reversible electrodes. Silver-silver chloride electrodes are generally used because they are stable and easy to prepare. The measuring and the reference Cl⁻ (aq) | AgCl (s) | Ag (s) electrodes for the patch clamp measurements were manufactured by chlorinating two silver wires, with diameters of 250 µm and 640 µm, respectively. The silver wires has silver content up to 99.9% and are made by Science Products (Hofheim, Germany).

In order to prepare the measuring and reference electrodes, the silver wires were scraped with fine sandpaper in order to remove a thin AgO coating, then cut to a length of ca. 4.5 cm. The wires were cleaned with methanol in order to remove any organic impurity. The dry wires were immersed in 20 mM HCl solution, and connected with the anode of the power supply unit. Another silver wire with a diameter of 640 µm was
Instruments and chemicals. Experimental details

attached to the cathode and immersed in the same electrolyte solution. The linear power supply unit was model LPS 301, a product of American Reliance Inc (Arcadia, California, USA). The electrolysis voltage was kept at constant 500 mV. The measuring electrode was chlorinated for 24 hours while the reference electrode was chlorinated for 48 hours.

\[
\begin{align*}
\text{Anode:} & \quad 2 \text{Ag(s)} + 2 \text{Cl}^- (a) \rightarrow 2\text{AgCl (s)} + 2 e^- (\text{Ag}) \\
\text{Cathode:} & \quad 2 \text{H}_3\text{O}^+ (aq) + 2 e^- (\text{Ag}) \rightarrow \text{H}_2 (g) + 2 \text{H}_2\text{O (l)}
\end{align*}
\]

The two above reactions occurred at the anode and the cathode, respectively. Evenly chlorinated electrodes with a dark grey AgCl coating were obtained and used in our experiments for about one week before replaced by new Cl\(^-\) (aq) | AgCl (s) | Ag (s) electrodes.

4.1.2 UV-VIS system

All the irradiation experiments were carried out using a 100 W high-pressure mercury lamp (Osram HBO 103/2, housing from Amko). The E-Z photoisomerisation was monitored using a Perkin-Elmer spectrometer model Lambda 19. The UV irradiation was achieved by the use of a band-pass filter (295 nm < \(\lambda\) < 400 nm). Visible radiation was obtained by the use of a longwave-pass filter (\(\lambda\) > 475 nm).

4.2 CHEMICALS, MATERIALS AND ANALYSIS

Soybean lecithin, type S-75, used to create the lipid bilayer was a product of Lipoid GmbH (Ludwigshafen, Germany). PET polymer with (RoTrac\textsuperscript{®}) microstructure was a product of Oxyphen GmbH (Grosserkmannsdorf, Germany).

All the chemicals and reagents used for patch clamp measurements were of analytical grade and were used as received. Cholesterol (> 99%), CsCl (> 99.5%), KCl
Instruments and chemicals. Experimental details

( > 99.5 %), NaCl ( > 99.5 %), hexadecane ( > 99 %), n-decane ( > 98 %) and squalene ( > 99 %) were purchased from Fluka Biochemika AG (Munich, Germany). Standard patch pipettes were obtained from Science Products GmbH (Hofheim, Germany). Ultrapure water was used for all the measurements of the artificial ion channels.

Methylresorcin, dodecanal, bromochloromethane, n-bromosuccinimide and 4-aminoazobenzene were used without further purification as received from Fluka and Aldrich. Solvents were dried by standard procedures. Analytical thin-layer chromatography (TLC) was performed on commercial Merck plates coated with silica gel 60 F 254 (0.25 mm thick). Merck Kieselgel 60 (0.040 - 0.063 mm) was used to prepare the silica gel for thin layer chromatography.

Nuclear magnetic resonance (NMR) spectroscopy was performed on a Bruker AC-300P (300.1 MHz) equipped with a variable temperature controller or in a Bruker DRX 500 (500.1 MHz), using deuterated chloroform (CDCl₃) or 1,1,2,2-tetrachloroethane-d₂ (TCL) as solvent and the internal solvent peak as the reference. The chemical shifts are expressed in ppm using TMS as the internal standard. Matrix assisted laser desorption ionisation (MALDI) spectra were obtained by Kratos Kompakt II mass spectrometer, using 1,8,9-trihydroxyanthracene (Dithranol) or 4-nitro-anilin (4-NA) as matrix.

4.3 EXPERIMENTAL DETAILS

4.3.1 UV-VIS experiments

The E-Z isomerisations of gated calixresorcinarene were performed in acetone solution. All the irradiation experiments were made at room temperature, using a thermostated cell compartment. Solutions of the analytes were prepared with a concentration of 1x10⁻⁵ mol/L and kept in the dark before the irradiation experiments in order to achieve
the E conformation. The solutions were continuously mixture during all the irradiation procedures. The distance between the lamp and the sample was about 20 cm.

4.3.2 Patch clamp experiments

Our artificial ion channels were incorporated into a porous polymer membrane\(^{136}\). Lipid bilayers were formed in a porous membrane by the painting technique. The lipid bilayer was formed from a mixture of 15mg/mL soybean lecithin, type S75 in squalene with 70/30 molar ratio lipid/cholesterol. Microporous PET polymeric micropores (pore diameter: 10\(\mu\)m, thickness: 16 \(\mu\)m, porosity: 15 \%) was used as the polymer matrix. The porous polymer matrix is key for a successful integration of ion channels compounds that directly influences the lipid bilayer fluidity and its mechanical and electrical stability. Scanning electron microscope (SEM) studies reveal very smooth edges and circular shapes of the pores. The diameters of the micropores obtained by chemical etching are very similar. They do not vary more than 10 \% and are sufficiently large to incorporate the ion channels and to provide the necessary area ratio of bilayer to annulus\(^{137}\). The components of the lipid solution also have an important influence on the fluidity of the lipid bilayers and on the stability of ion channels incorporation. Lipid/cholesterol mixture in a 70/30 ratio had been previously found to yield a stable bilayer for 1.5 hours and relatively good fluidity and activity for the incorporation of ion channels compound. More cholesterol added restrict the rapid movements of the hydrocarbon chains in lipid molecules\(^{137}\). The lipid bilayer will be formed across the pores of the matrix.

The suspension of phospholipid in n-squalene solution was applied over the porous polymer, which itself was attached to a PTFE sample chamber with a small hole of 0.6 mm diameter (Figure 15). The small pore covered by polymer separates two aqueous compartments of the chamber: the measuring compartment and reference compartment, each about 1 mL in volume. Both compartments of the chamber were filled with 0.5 M KCl electrolyte and equipped with Ag/AgCl electrodes (the measuring
electrode into the measuring chamber, and the reference electrode into the reference chamber).

**Figure 15**: Patch clamp chamber and lipid bilayer formation in the micropores of a polymer matrix
The basic approach to measure small ionic currents in the picoampere range through single channels requires a low-noise recording technique. Before every experiment, all noise sources must be checked carefully, including the air pressure of the vibration isolation table.

Some preparation must be done in advance before starting the experiment:

- Cleaning the pipette holder: The pipette holder must be removed from the measuring electrode after each experiment and washed with ultra-pure water. Then the pipette holder need to be ultrasonically cleaned for about 15 minutes and after then washed with ethanol and chloroform before drying.

- Cleaning the sample chamber: the sample chamber must be first washed with water and ultra-pure water to remove all the salts. The gel used for sealing the polymer matrix on the chamber must be removed using a stainless-steel slice. Before being washed several times with ultra-pure water, the chamber need to be ultrasonically cleaned for another 15 minutes. The cleaned chamber was washed again with ethanol and chloroform before drying.

- The polymer matrix was sealed to the bottom of the clean and dry chamber and stored for about 12 hours in a dust-proof container.

- Bath solution: all bath solutions, as well as ultra-pure water were filtered by a 0.02 μm filter before the experiments.

- Lipid solution: the dry lipids were stored in a deep freezer to prevent decomposition. Lipid solutions prepared in n-squalene were stored at 4°C in a refrigerator. For the patch clamp measurements, such a lipid solution can be used only for one week.

- The measuring and the reference electrodes were renewed every month.

- The EPC9 amplifier was calibrated every month in order to avoid baseline drift. The noise level was measured before every experiment.
For the conductivity measurements we used a KCl salt solution with a concentration about 0.5 mol/L. Approximately 1 mL of KCl solution was added to the measuring compartment of the chamber and about 1 mL of the same solution was also added to the reference compartment. The procedure for noise measurements procedure was started before every experiment as follows:

- The EPC-9 amplifier button was selected in order to display the root mean square (rms) noise current.
- The frequency bands were selected by filter 1 as 10 kHz and filter 2 as 3 kHz.
- The standard gain control was setting at 50mV/pA, for the noise measurements
- EPC-9 with nothing connected to the input should show noise (rms) usually 80 - 100 fA (filter 2 as 3 kHz); 90 - 120 fA (filter 2: 5 kHz). When the reading values are higher than these, the C-fast control must be modified.
- After the pipette holder was installed and the pipette was filled with bath solution, the noise reading should be about 100 – 130 fA.
- After the pipette tip was slowly immersed into bath solution, the noise (rms) reading should be less than 160 fA. If the noise reading is much higher than this value, examination of the pipette holder, glass pipette and electrode is needed.

1. reducing noise from pipette holder:
   - using manufactured low dielectric materials (PTFE and polycarbonate),
   - handling using a tool instead of hands in order to avoid fingerprint grease,
   - cleaning with ethanol and ultra-water following by drying blowing dry nitrogen or air through the holder.

2. reducing noise from glass pipette:
   - using borosilicate glass or aluminosilicate glass,
   - using a fresh pipette for every measurements,
   - dipping the pipette tip into the solution as slowly as possible,
- keeping the solution level below than the middle level of the patch pipette,
- using filtered solution in the chamber as well as in the pipette,
- keeping the end of the pipette dry and clear of any fingerprint grease and solution

3. reducing the noise from electrode (Ag/AgCl wire):
- only the end of the wire, which will be in contact with the pipette filling solution, should be chlorinated,
- the other end of the wire should be kept clean to ensure a good contact with the pin at the back of the holder, which should be not scratched (the chloride coating via fitting the pipette into the holder),
- the pipette-filling solution should not be in contact with the bare silver wire; an offset potential between the salt that fill the pipette solution and the silver wire leads to the membrane potential set on the patch clamp amplifier.

The next step is the formation of the lipid bilayer. A small amount of lipid solution was painted over the dry surface region of the polymer matrix using a special brush. The preliminary lipid multilayers were destroyed by applying negative pressure using the pump. The lipid solution around the micro pores will thus be removed. However, a part of the annulus still remains along the walls of these micro pores, which will act as a reservoir for the succeeding construction of the lipid bilayer. Previous experience indicate that this sort of annulus is absolutely required in order to obtain a stable bilayer\textsuperscript{138}.

In the next step the pipette was introduced into the measuring compartment of the chamber and the pipette resistance was examined by applying voltages from $-10 \text{ mV}$ to $+10 \text{ mV}$. In the following step, a small amount of lipid solution is again drawn across the aperture region using the brush. The film will be initially several µm thick and it will be in equilibrium with a much larger annulus formed at the perimeter of the micro
pores (the Plateau-Gibbs border). Then the film thins spontaneously to form the lipid bilayer.

The current characteristics during the thinning of the membrane were continuously monitored until a stable current baseline was achieved.
After the lipid bilayer was formed across the pores of the membrane, the mechanical and electrical properties of the lipid bilayer were monitored for 10 min before the addition of calix[4]resorcinarene into the two compartments of the chamber. The leakage of the bilayers was examined under 100 mV and 200 mV command voltage.

Independent patch clamp experiments were performed using different concentrations (10^{-8} mol/L, 10^{-12} mol/L) of compounds in acetone or chloroform solution. The compounds were added into the measuring compartment and symmetrically into the reference compartment of the chamber by 5 µL steps from lower concentration to higher concentration until stable single-channel signals across the lipid bilayer were observed on the patch clamp. The channel conductance of tetramethyl-cavitand body was examined in a symmetrical aqueous solution of KCl (0.5 mol/L) under different electrical potentials (between –200 mV and +200 mV, in 25 mV steps). The electrolyte solutions used for experiments were 0.5 mol/L KCl (NaCl, CsCl, CaCl_2) added into the both (measuring and reference) compartments of the chamber. Every compound was investigated via patch clamp in four independent runs. All experiments were carried out by room temperature.
5 SYNTHESIS OF AZOBENZENE SUBSTITUTED CALIX[4]RESORCINARENES

Initial attempts to design light-gated molecular switches included the incorporation of (i) olefinic and (ii) azo groups. Synthesis of light-gated calix[4]resorcinarene cavitands based on olefinic groups did not prove very promising because of photoisomerisation side reactions\textsuperscript{139} hence we focused on light-responsive azo groups within the substituents $R_5$ to $R_8$ (Figure 16).

![Chemical Structure](image.png)

**Figure 16:** Target compounds for upper rim substituted light-gated calix[4]resorcinarenes cavitand:

- 5 - tetrakis(p-phenylazophenylaminomethyl)-cavitand;
- 6 - 23-methyl-tris(5,11,17-p-phenylazophenylaminomethyl)-cavitand;
- 7 - 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand;
- 8 - 11,17,23-trimethyl-(5-p-phenylazophenylaminomethyl)-cavitand
The synthesis of the light-gated calix[4]resorcinarene cavitands is described below. The description is organized according to the necessary major synthetic steps. All intermediate and final products were characterised and their structures were confirmed by NMR and mass spectrometry.

At first the compound calix[4]resorcinarene(OH, C_{11}H_{23}) type (right compound in Figure 17) was synthesized via acid-catalysed reaction of 2 methylresorcin (left compound in Figure 17) with dodecanal (C_{12}H_{24}O). The synthesis of calix[4]resorcinarene(OH, C_{11}H_{23}) followed the experimental conditions given for a similar compound\textsuperscript{140}.

![Figure 17: Acid-catalysed reaction for synthesis of calix[4]resorcinarene (OH, C_{11}H_{23}, right compound) from 2 methylresorcin (left compound) and dodecanal (C_{12}H_{24}O)](image)

**General procedure for the preparation of calix[4]resorcinarene (OH, C_{11}H_{23})**

In a 250 mL three-necked round bottom flask, a solution of 2-methylresorcin (0.18 mol) in 75 mL ethanol was acidified with 24 mL HCl and cooled to 0°C. Dodecanal (0.18 mol) was dissolved in 50 mL ethanol. The dodecanal solution was added dropwise over a period of two hours to the solution of 2-methylresorcin while stirring at 0°C under nitrogen. The resulting brown solution was allowed to warm slowly to 25°C and
then heated to 75°C for 48 hours. During this time, the colour changed to red, and a light orange precipitate separated. The pale orange precipitate was washed repeatedly with cold methanol and was dried under vacuum. The reaction yield was 56%.

Spectroscopic data

$^1$H NMR (300 MHz, CDCl$_3$, rt): $\delta$ (ppm) = 0.89 (t, 2H, CH$_3$, $J = 6.9$ Hz), 1.3 (m, 18H, (CH$_2$)$_9$), 2.29 (m, 8H, CH$_2$), 4.35 (t, 4H, CH, $J = 6.9$ Hz), 7.40 (s, 4H, Ar-H), 8.00 (s, 8H, OH)

MALDI TOF-MS (Dithranol): calcd for C$_{76}$H$_{120}$O$_8$: m/z =1161.80 [M]$^+$; found: m/z =1185 [M+Na]$^+$.

In the next step the synthesis of tetramethyl-cavitand compound was performed.

Figure 18: Synthesis of tetramethyl-cavitand compound (right) from calix[4]resorcinarene (OH, C$_{11}$H$_{23}$) type (left)

Tetramethyl-cavitand was obtained according to known procedures$^{141,142}$. By reacting calix[4]resorcinarene(OH, C$_{11}$H$_{23}$) (left compound in Figure 18) with the equivalent of CH$_2$BrCl in N,N-dymethylformamide (DMF) and K$_2$CO$_3$ the bridging of the neighbouring hydroxyl groups was achieved. This resulted in rigidifying the molecule to give tetramethyl-cavitand (right compound in Figure 18).

Via this reaction, the network of hydrogen bond among the hydroxyl groups of calix[4]resorcinarene(OH, C$_{11}$H$_{23}$) was replaced by covalent bonds. The resulting cavitand has an extremely rigid conformation compared to the parent calix[4]resorcinarene(OH, C$_{11}$H$_{23}$).

**General procedure for the preparation of tetramethyl-cavitand**

In a three-necked round bottom flask connected with a reflux condenser, a solution of 27 mmol calixarene(OH, C$_{11}$H$_{23}$) type in 250 mL DMF was added dropwise over a period of two hours to a suspension of 0.54 mol K$_2$CO$_3$ and 0.43 mol CH$_2$BrCl. When the addition was complete, the pink solution was stirred mechanically at 70° C under a nitrogen atmosphere for 4 days. The reaction solution was then filtered and the solvent was removed by rotary evaporation. The resulting red oil was dissolved in CH$_2$Cl$_2$ (250 mL) and 2N HCl (150 mL). The organic layer was separated, washed with 2N HCl (3x100 mL), H$_2$O (100 mL) and dried over MgSO$_4$. The organic layer was eluted from a short silica column (2 cm) with CH$_2$Cl$_2$ and the solvent was removed in vacuum to afford a cream coloured product (62%).

**Spectroscopic data**

$^1$H NMR (300 MHz, CDCl$_3$, rt): $\delta$ (ppm) = 0.89 (t, 12H, CH$_3$, J = 6.87 Hz), 1.32 (m, 18H, (CH$_2$)$_9$), 1.99 (s, 12H, CH$_3$), 2.20 (m, 8H, CH$_2$), 4.25 (d, 4H, OCH$_2$, J = 6.87 Hz), 4.75 (t, 4H, CH, J = 8 Hz), 5.90 (d, 4H, OCH$_2$, J = 6.87 Hz), 6.99 (s, 4H, Ar-H)

MALDI TOF-MS (Dithranol): calcd for C$_{80}$H$_{120}$O$_8$: $m/z$ =1208 [M]$^+$; found: $m/z$ =1231 [M+Na]$^+$.

In the next step the synthesis of tetrakis(bromomethyl)-cavitand 1, tris(bromomethyl)-cavitand 2, bis(bromomethyl)-cavitand 3, and bromomethyl-cavitand 4 (right compounds in Figure 19) was performed.

Bromination of methylcavitand$^{143}$ (left compound in Figure 19) with N-bromo-succinimide (NBS) in CH$_2$Cl$_2$ and with 2,2'-azobisisobutyronitrile (AIBN) as catalyst
gave tetrakis(bromomethyl)-cavitand 1, tris(bromomethyl)-cavitand 2, bis(bromo-
methyl)-cavitand 3, bromomethyl-cavitand 4 as products (right compounds in Figure
19).

**Figure 19:** Synthesis of tetrakis(bromomethyl)-cavitand 1, tris(bromomethyl)-cavitand
2, bis(bromomethyl)-cavitand 3, and bromomethyl-cavitand 4 (right compounds) from
tetramethyl-cavitand (left compound):

1 - tetrakis(bromomethyl)-cavitand;
2 - tris(bromomethyl)-cavitand;
3 - bis(bromomethyl)-cavitand;
4 - bromomethyl-cavitand

**General procedure for the preparation of tetrakis(bromomethyl)-cavitand 1**

A solution of 4.5 mmol tetramethyl-cavitand, four equivalents NBS (20.2 mmol) and a
catalytic AIBN were refluxed in 300 mL chloroform. The mixture was heated under
nitrogen to 80°C and stirred for six days. The reaction mixture was cooled to room
temperature and the precipitate was removed by filtration. The solvent from the organic
layer was removed under low pressure and a dark orange oil containing a mixture of
tetrakis(bromomethyl)-cavitand 1, tris(bromomethyl)-cavitand 2, bis(bromomethyl)-cavitand 3, bromomethyl-cavitand 4 as products was obtained.

These four synthesised cavitand types were used as intermediate compounds to obtain upper rim substituted calix[4]resorcinarene cavitands with different numbers of azobenzene groups: tetrakis(p-phenylazophenylaminomethyl)-cavitand 5; 23-methyl-tris(5,11,17-p-phenylazophenylaminomethyl)-cavitand 6; 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand 7, 11,17,23-trimethyl-(5-p-phenyl-azophenylaminomethyl)-cavitand 8 (right compounds in Figure 20)\textsuperscript{144}. The intermediate mixture of tetrakis(bromomethyl)-cavitand 1, tris(bromomethyl)-cavitand 2, bis(bromomethyl)-cavitand 3, bromomethyl-cavitand 4 tetrakis(bromomethyl)-cavitand 1 was reacted with 4-aminoazobenzene. The mixture was used without purification, due to instability of the products on different column materials (e.g., silica and aluminium oxide).

\begin{figure}[h!]
\centering
\includegraphics[width=\textwidth]{figure.png}
\caption{Synthesis of upper rim gated calix[4]resorcinarenes cavitand :}
5 - tetrakis(p-phenylazophenylaminomethyl)-cavitand;
6 - 23-methyl-tris(5,11,17-p-phenylazophenylaminomethyl)-cavitand;
7 - 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand;
8 - 11,17,23-trimethyl-(5-p-phenylazophenylaminomethyl)-cavitand

General procedure for the preparation of tetrakis(p-phenylazophenylaminomethyl)-cavitand 5

0.6 mmol intermediate mixture of tetrakis(bromomethyl)-cavitand 1, tris(bromomethyl)-cavitand 2, bis(bromomethyl)-cavitand 3, bromomethyl-cavitand 4 and 4-aminoazobenzene were weighed into a 100 mL dry three-necked round bottom flask connected to a 50 mL dropping funnel. 30 mL dry DMF and 6 mmol anhydrous potassium carbonate were added and the mixture was stirred at room temperature. During stirring, 3 mmol 4-aminoazobenzene dissolved in 30 mL dry DMF were added dropwise to the mixture over a period of 2 h. After connecting an argon balloon, the mixture was heated to 45°C and kept under vigorous stirring 5 days. Then, the solvent was removed under high vacuum and 1:1 mixture of methylene chloride-water was added. The aqueous solution was removed with a separatory funnel. The crude product was purified by column chromatography (SiO₂, pentane - dichloromethane (1:1) to afford the tetrakis-(p-phenylazophenylaminomethyl)-cavitand 5, 23-methyl-tris(5,11,17-p-phenylazophenylaminomethyl)-cavitand 6, 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand 7 and 11,17,23-trimethyl-(5-p-phenylazophenylaminomethyl)-cavitand 8\(^\text{144}\).

Spectroscopic data of compounds\(^\text{144}\):

**Tetrakis(p-phenylazophenylaminomethyl)-cavitand 5:**

\(^1\)H NMR (500 MHz, CDCl₃, rt): \(\delta\) (ppm) = 0.86 (t, 12H, CH₃, J = 6.74 Hz), 1.21 (m, 64H, CH₂CH₂ ), 1.45 (s, 8H, CH₂CH₂), 2.21 (m, 8H, CH₂CH₂CH), 4.16 (d, 8H, CH₂NH), 4.29 (br s, 4H, NH), 4.35 (d, 4H, OCH₂O, J = 6.87 Hz), 4.76 (t, 4H, CHCH₂, J = 8.04), 5.96 (d, 4H, OCH₂O, J = 6.81 Hz), 6.76 (d, 8H, ArH, J = 8.68), 7.14 (s, 4H, ArH ), 7.38 (t, 4H, ArH, J = 8.68), 7.48 (t, 8H, ArH , J = 7.55), 7.85 (t, 16H, Ar H, J = 7.48).

13C NMR (125.7 MHz, CDCl3, rt): δ (ppm) = 14.12 (CH3), 22.68, 27.88, 29.83, 31.93 (CH2), 37.01 (CHCH2), 37.90 (ArCH2NH), 99.67 (OCH2O), 113.50, 120.8, 122.30, 125.22, 129.08, 129.71 (Caryl), 139.48, 145.96, 148.12, 150.04, 152.99, 153.56 (Caryl).


23-Methyl-tris(5,11,17-p -phenylazophenylaminomethyl)-cavitand 6:

1H NMR (500 MHz, CDCl3, rt): δ (ppm) = 0.87 (t, 12H, CH3, J = 6.94 Hz), 1.26 (m, 72H, CH2), 1.96 (m, 3H, ArCH3), 2.22 (br s, 8H, CH2CH2CH), 4.15 (t, 3H, NH), 4.2 (s, 6H, ArCH2NH), 4.29 (d, 2H, OCH2O, J = 6.91), 4.39 (d, 2H, OCH2O, J = 6.91), 4.74 (m, 4H, CHCH2), 5.97 (d, 2H, OCH2O, J = 6.93), 6.12 (d, 2H, OCH2O, J = 6.89), 6.76 (t, 3H, ArH, J = 8.82), 6.97 (s, 2H, ArH), 7.12 (s, 2H, ArH), 7.37 (t, 6H, ArH, J = 7.2), 7.46 (m, 6H, ArH), 7.82 (m, 12H, ArH).

13C NMR(125.7 MHz, CDCl3, rt): δ (ppm) =12.02 (ArCH3), 14.12(CH3), 22.60, 27.88, 29.70, 31.92 (CH2), 37.02(CHCH2), 38.08 (ArCH2NH), 99.61 (OCH2O), 112.96, 113.32, 122.42, 125.22, 128.96, 129.69 (Caryl), 138.94, 145.02, 148.08, 150.24, 152.98, 153.7 (Caryl).

MALDI TOF-MS(Dithranol): calcd for C116H147N9O8: m/z =1797 [M]+; found: m/z =1800 [M+H]+.

11,23-Dimethyl-bis(5,17-p -phenylazophenylaminomethyl)-cavitand 7:

1H NMR (500 MHz, CDCl3, rt): δ (ppm) = 0.83 (br s, 12H, CH3), 1.23 (m, 72H, CH2), 1.97 (d, 6H, CH3Ar, J =11.31), 2.19 (br s, 8H, CH2CH2CH), 3.98 (br s, 2H, NH), 4.19 (d, 4H, CH2NH), 4.31 (d, 4H, OCH2O, J = 6.89), 4.77 (br s, 4H, CHCH2), 5.94 (m, 4H,

OCH₂O), 6.75 (t, 4H, ArH, J = 8.38), 6.98 (s, 2H, ArH ), 7.12 (s, 2H, ArH ), 7.39 (m, 2H, ArH ), 7.47 (m, 4H, ArH ), 7.83 (m, 8H, ArH ).

¹³C NMR (125.7 MHz, CDCl₃, rt): δ (ppm)=12.02 (ArCH₃), 14.12 (CH₃), 22.68, 27.90, 29.82, 31.92 (CH₂), 37.00 (CHCH₂), 37.94 (CH₂NH), 99.02 (OCH₂O), 113.60, 119.62, 122.32, 125.09, 128.94, 129.78 (Caryl), 141.00, 147.02, 149.52, 152.94, 153.04, 153.96 (Caryl).

MALDI TOF-MS (Dithranol): calcd for C₁₀₄H₁₃₈N₆O₈: m/z =1600.24 [M]+; found: m/z =1602 [M+H]⁺.

¹¹,¹⁷,²³-Trimethyl-(5-p -phenylazophenylaminomethyl)-cavitand 8 :

¹H NMR (500 MHz, CDCl₃, rt): δ (ppm)=0.86 (t, 12H, CH₃, J =6.91 Hz), 1.25 (m, 72H, CH₂), 1.94(m, 9H, ArCH₃), 2.20 (br s, 8H, CH₂CH₂, CH), 4.06 (m, 2H, NH ), 4.20 (d, 2H, CH₂NH, J =8.04), 4.23 (m, 4H, OCH₂OH), 4.70 (d, 4H, CHCH₂ ), 5.89 (m, 4H, OCH₂O), 6.74 (t, 2H, Ar H , J =8.56), 6.99 (s, 2H, ArH ), 7.10 (s, 2H, ArH ), 7.38 (t, 2H, ArH, J =7.37), 7.46 (t, 1H, ArH , J =7.67), 7.82 (m, 3H, ArH ).

¹³C NMR (125.7 MHz, CDCl₃, rt): δ (ppm) = 12.02 (ArCH₃), 14.12 (CH₃), 22.61, 27.58, 29.79, 31.92 (CH₂), 36.95 (CHCH₂), 37.58 (ArCH₂NH), 99.67 (OCH₂O), 113.07, 113.64, 122.30, 125.22, 129.08, 129.71 (Caryl), 139.48, 147.00, 148.15, 150.14, 152.89, 153.01 (Caryl).

MALDI TOF-MS (Dithranol): calcd for C₉₂H₁₂₉N₃O₈: m/z =1405.02 [M]+; found: m/z =1405 [M]⁺.
6 PHOTOISOMERISATION EXPERIMENTS

In order to prove the photoisomerisation capability of synthesised light-gated calix[4]resorcinarene we have performed UV-VIS experiments. Geometrical isomerisation of light-gated calix[4]resorcinarene by irradiation with light of different wavelengths should induce significant structural changes. The E, Z isomers of this molecule will switch from one structure to another under photoexcitation, with the structural change occurring during the electronically excited state. The E- Z isomerisation will be observed by differences in the absorption spectra. First we present our photoisomerisation results for the single chromophore system: the compound with only one azobenzene substituent (11,17,23-trimethyl-(5-p-phenylazophenylaminomethyl)-cavitand 8), followed by the description of the behaviour of multiple azobenzene chromophore systems: the compound with two azobenzene substituents (23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand 7) and with four azobenzene substituents (tetrakis(p-phenylazophenylaminomethyl)-cavitand 5).

6.1 SINGLE-CROMOPHORE SYSTEM

In case of 11,17,23-trimethyl-(5-p-phenylazophenylaminomethyl)-cavitand 8 (calix[4]resorcinarene with one azobenzene substituents in upper rim, Figure 20) in acetone solution, the two isomers (Figure 21) exhibit well-separated absorption bands in the UV-Vis range. The E configuration of 11,17,23-trimethyl-(5-p-phenylazophenylaminomethyl)-cavitand 8 exhibits a large absorbance peak at $\lambda_{\text{max}} = 385$ nm (Figure 22) that corresponds to a $\pi-\pi^*$ transition. Main features of the absorbance spectrum of the Z configuration are a very weak band in the VIS range and an intense band in the UV range. The absorbance maximum of the very broad band in the VIS range at about $\lambda_{\text{max}} = 523$ nm (Figure 22) is hardly visible because the n-$\pi^*$ transition that exhibits the band is forbidden.
Photoisomerisation experiments

**Figure 21:** Optical isomers of 11,17,23-trimethyl-(5-p-phenylazophenyl aminometyl)-cavitand 8, E state (left) and Z state (right)

The n-orbitals do not overlap well with the \( \pi^* \) orbital, thus the probability of excitation is very small and correspondingly the magnitude of the molar absorptivity is very small. In addition, the Z configuration of 11,17,23-trimethyl-(5-p-phenylazophenylamino-\( \text{metyl})\)-cavitand 8 also exhibits an absorbance band in the UV range (\( \lambda < 330 \) nm). This band cannot be observed on our recorded spectra because it is covered by the strong absorbance of the solvent acetone. Acetone had to be used for measuring the UV-VIS spectra because our compounds are not soluble in solvents which are transparent below 330 nm.

The intense band of the E isomer (in UV range) is well separated from the two Z bands just described. The different peaks with large absorbance of E isomer (observed in Figure 22) and the double absorbance peaks of Z isomer of 11,17,23-trimethyl-(5-p-phenylazophenyl aminometyl)-cavitand 8 are conform with the other reported azo derivatives\textsuperscript{145,146}. 
Photoisomerisation experiments

**Figure 22:** E,Z-isomerisation of 11,17,23-trimethyl-(5-p-phenylazophenyl aminomethyl)-cavitand 8 in acetone solution

Upon UV light irradiation (filter 295 nm < \( \lambda < 400 \) nm) the band of the E isomer with \( \lambda_{\text{max}} = 385 \) nm (spectrum 2 in Figure 22) considerably decreased and that of the Z isomer with \( \lambda_{\text{max}} = 523 \) nm (spectrum 3 in Figure 22) slightly increased\(^{147}\). This indicates the isomerisation of the E form to the Z form after 5 minutes irradiation. The Z isomer was irradiated in the VIS range (filter \( \lambda > 475 \) nm). The Z isomer recovered to the E isomer (spectrum 3 in Figure 22) after 10 minutes irradiation. The fact that the peak at 385 nm obtained after irradiation (curve 3 in Figure 22) exceeds that for curve 1 (in Figure 22) indicates that both conformers exist in thermal equilibrium before irradiation.
In case of 11,17,23-trimethyl-(5-p-phenylazophenylaminometyl)-cavitand 8 in acetone solution, two isosbestic points were recorded in the absorption spectra at $\lambda = 346$ nm and at $\lambda = 454$ nm. The coexistence of two absorbing species, in our case the E and Z isomers of the azobenzene moiety, is usually indicated by only one isosbestic point. The occurrence of two isosbestic points in Figure 22 is due to the band doublet of the Z isomer surrounding the absorbance band of the E isomer. During the irradiation process of 11,17,23-trimethyl-(5-p-phenylazophenylaminometyl)-cavitand 8, both absorbance bands of the Z isomer cross the absorbance of the E isomer resulting in two isosbestic points. The presence of two isosbestic points in the electronic spectra of our compound, is in full agreement with other reports on azobenzene derivatives\textsuperscript{46,48}.

### 6.2 Multi-Chromophore System

In case of 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand 7 (calix[4]resorcinarene with two azobenzene substituents in upper rim, Figure 16), the EE, ZZ isomerisation was performed in acetone solution. As explained earlier, acetone had to be chosen because of the very restricted solubility of the investigated compounds. The EE isomer of 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand 7 has both azobenzene groups on the upper rim in E state, while the ZZ isomer of 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand 7 has both azobenzene groups on the upper rim in Z state. The EE isomer of 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand 7 exhibit an intense band in UV range at $\lambda_{\text{max}} = 385$ nm (Figure 23). The ZZ configuration exhibit a simultaneously double absorbance spectra: a very weak band in VIS range at about $\lambda_{\text{max}} = 523$ nm and one intense band in UV range. The absorbance peak of the ZZ isomer in UV range at $\lambda < 330$ nm cannot be observed on our recorded spectra due to the acetone absorbance limitation.
Figure 23: EE,ZZ-isomerisation of 11,23-Dimethyl-bis(5,17-p-phenylazophenyl-aminomethyl)-cavitand 7 in acetone solution

Spectrum 1 in Figure 23 represents the thermodynamically favoured EE conformation. Upon irradiation with UV light (filter $295 \text{ nm} < \lambda < 400 \text{ nm}$) the two azobenzene moieties are converted to the ZZ form (spectrum 2 after 3 minutes irradiation and spectrum 3 after 5 minutes irradiation). Both states were interconverted photochemically using a filter $295 \text{ nm} < \lambda < 400 \text{ nm}$ for EE-ZZ transformation (spectrum 2 after 3 minutes irradiation and spectrum 3 after 5 minutes irradiation) and a filter $\lambda > 475 \text{ nm}$ for the back reaction (spectrum 4 after 10 minutes irradiation). Two isosbestic points were observed, one at $\lambda = 347 \text{ nm}$ and one at $\lambda = 452 \text{ nm}$. In case of the single-chromophore system based on 11,17,23-trimethyl-(5-p-phenylazophenyl-aminomethyl)-cavitand 8 we have observed the presence of two isosbestic points. As explained earlier, the presence of two isosbestic points in a single-chromophore system indicate strong evidence for a two state system (E and Z), which are in chemical
equilibrium with each other. In case of two-chromophore system 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand 7, two isosbestic points were detected on electronical spectra. Similar to the single-chromophore system, the presence of two isosbestic points on a two chromophore system indicate strong evidences for only two absorbing species (EE and ZZ). The coexistence of two absorbing species, in our case the EE and ZZ isomers of the azobenzene moiety, is usually indicated by only one isosbestic point. The occurrence of two isosbestic points (in Figure 23) is due to the band doublett of the ZZ isomer surrounding the absorbance band of the EE isomer. During the irradiation process of 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand 7, both absorbance bands of the ZZ isomer cross the absorbance of the EE isomer resulting in two isosbestic points. Moreover, during the photoconversion, the transient electronic spectra of 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand 7 does not indicate the intermediate EZ state, but indicate only EE and ZZ forms. Such a multiple system will indicate the intermediate EZ state in electronical spectra if there will be intramolecular dipol-dipol interactions between the two azobenzene moieties from the upper rim of calix[4]resorcinarene. On the UV-VIS spectra, this interactions will cause a spectral wavelength shift for the EZ absorbing species (bathochromism or hypsochromism). Due to the shifted absorbance of the EZ isomer, no isosbestic point occurs anymore.

During our experiments, no absorption spectral shift occurs as we can conclude from the appearance of the two isosbestic points. The electronical spectra indicate only two photointerconvertible states that contribute to the transient spectra, EE and ZZ forms. This means that both azobenzene chromophores does not exhibit a dipol-dipol interaction and the system behave like the single-chromophore system during the isomerisation process.

In case of tetrakis(p-phenylazophenylaminomethyl)-cavitand 5 (calix[4]resorcinarene with four azobenzene substituents in upper rim, Figure 16), the transient spectra of the EEEE-ZZZZ isomerisation become very complex. The ZZZZ isomer of tetrakis(p-phenylazophenylamino-methyl)-cavitand 5 has all four azobenzene groups in Z form, while the EEEE isomer has all four azobenzene groups in E form. The possible
intermediate states of photoconversion are: ZZZE, ZZEE, ZEEE, ZEZE. In case of ZZZE isomer of tetrakis(p-phenylazophenyl-aminomethyl)-cavitand 5, three azobenzene groups are in Z state and one azobenzene group is in E state, while in case of ZZEE isomer of tetrakis(p-phenylazophenylaminomethyl)-cavitand 5, two neighbouring azobenzene groups are in Z state and other two neighbouring azobenzene groups are in E state. The ZEEE form of tetrakis(p-phenylazophenyl-aminomethyl)-cavitand 5 has one azobenzene group in Z state and three azobenzene groups in E state. The ZEZE form of tetrakis(p-phenylazophenylaminomethyl)-cavitand 5 has two opposite azobenzene groups in Z state and other two opposite azobenzene groups in E state. On this case, the photoconversion electronic spectra does not behave anymore similar to that of the single-chromophore system. During the photoirradiation process of tetrakis(p-phenylazophenylaminomethyl)-cavitand 5, beside the pure EEEE and ZZZZ forms we get also an intermediate state. This we can conclude since no isosbestic point occurs on the electronical spectra. The presence of a shifted absorbance indicate a dipol-dipol interaction between the intramolecular azobenzene chromophore moieties which could be ZZZE, ZZEE, ZEEE or ZEZE, forms that could not be discriminated because of the high complexity multi-chrompophore system and the stochastic steric interaction process between the four azobenzene moieties (cf. 148).

The stability of 11,17,23-trimethyl-(5-p-phenylazophenyl aminomethyl)-cavitand 8, 11,23-Dimethyl-bis(5,17-p-phenylazophenylamino-methyl)-cavitand 7 and of tetrakis(p-phenylazophenylaminomethyl)-cavitand 5 were tested by extended UV irradiation. No changes in the UV spectra could be observed after 8 hours continuous irradiation using a 100 W high-pressure mercury lamp, which showed that the compounds remain intact during irradiation.

The lifetime of the Z-form of 11,17,23-trimethyl-(5-p-phenylazophenyl aminomethyl)-cavitand 8, of the ZZ-form of 11,23-Dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand 7 and of the ZZZZ-form of tetrakis(p-phenylazophenylaminomethyl)-cavitand 5 in the dark is about 2 hours. This result is in agreement with similar investigations of azobenzene derivatives (cf. 149). Because the E (respectively EE,
Photoisomerisation experiments

EEEE) ground state lies lower in energy than that of Z-isomer (respectively ZZ and ZZZZ), any Z (respectively ZZ, ZZZZ) species created return to E (respectively EE, EEEE) not only photochemically but also thermally.
7 INTEGRATION OF ARTIFICIAL ION CHANNELS INTO LIPID BILAYER

The synthesised compounds need to be integrated into a lipid bilayer and measured using the patch clamp system in order to assess the ion channel activity. Basic experiments were performed for different compounds to study the influence of the length of the molecules on artificial ion channel formation and the selectivity features.

7.1 THE INFLUENCE OF ALKYL CHAIN LENGTH. SELECTIVITY TESTS

Artificial channels based on gramicidin A type like calix[4]resorcinarene synthesised here can switch from "off" state to an "on" state once two molecules form a dimer that span the lipid bilayer. In order to prove how the length of alkyl chains attached to the lower rim of the calix[4]resorcinarene influence the ion channel formation several compounds; one type of calix[4]resorcinarene with long alkyl chains (R=C$_{11}$H$_{23}$) and one type of calix[4]resorcinarene with short alkyl chains (R=C$_{6}$H$_{13}$) were investigated by patch clamp.

Patch clamp system is also a powerful tool to detect lipid bilayer leaks. Lipid bilayer leaks (bilayer temporarily defects), non-selectively, allow the passage of ions through the lipid bilayer, whereas ion channels are selective and discriminate between different ions that are going through lipid bilayer. Using different type of ions we have performed basic selectivity measurements for these types of artificial ion channel.

- **Calix[4]resorcinarene type with long alkyl chains**

Tetramethyl-cavitand (Figure 24) that has long alkyl chains (R=C$_{11}$H$_{23}$) attached to the lower rim was investigated by patch clamp measurements. The compound in chloroform solution was added both into the measuring and into the reference compartments and permitted to integrate into the bilayer.
Both measuring and reference compartments were filled with the same aqueous electrolyte solution. The recorded patch clamp traces clearly indicate ion channel formation.

**Figure 24:** Tetramethyl-cavitand structure

In order to study the selectivity property of the tetramethyl-cavitand, a series of patch clamp experiments for this compound were performed using KCl, NaCl, CsCl or CaCl₂ as electrolyte.

**Tetramethyl-cavitand in KCl electrolytic solution**

The ion flux through artificial ion channels formed by tetramethyl-cavitand and the changes in the bilayer conductivity are observed as square-wave pulses with approximate constant amplitude (Figure 25). Negative short constant currents were recorded once the dimer is formed, and followed by closed period states achieved by the displacement of the molecules.
Integration of artificial ion channels into lipid bilayer

**Figure 25:** Patch clamp recording of tetramethyl-cavitand in acetone solution (10^-8 mol/L) under –150 mV in a symmetrical aqueous solution of KCl (0.5 mol/L)

**Tetramethyl-cavitand in NaCl electrolytic solution**

The experiments performed under NaCl electrolytic solution prove that tetramethyl-cavitand compound incorporate successfully into the lipid bilayer (Figure 26). The compound formed ion channels with short open and closed states, which permit the Na⁺ translocation with one conductance level.

**Figure 26:** Patch clamp recording of tetramethyl-cavitand in acetone solution under –150 mV in a symmetrical aqueous solution of NaCl (0.5 mol/L)
Tetramethyl-cavitand in CsCl electrolytic solution

The patch clamp traces indicate no channel activity. No electrical current is measured through the lipid bilayer regardless reasonable variations in concentrations. Moreover, no electrical current is measured through the lipid bilayer regardless different applied potentials (between -200 mV and +200 mV), which indicate that artificial ion channels formed by tetramethyl-cavitand are not permissive for Cs$^+$ ion. On the other hand, the complete absence of ion currents indicates the formation of a bilayer without permanent or intermediate leaks.

Tetramethyl-cavitand in CaCl$_2$ electrolytic solution

Tetramethyl-cavitand was also investigated using CaCl$_2$ electrolytic solution under different concentrations and different voltages. No electrical current was measured through the lipid bilayer (Figure 28), which indicates that tetramethyl-cavitand ion channels are not permissive for Ca$^{2+}$ ion as well.
Figure 28: Patch clamp recording of tetramethyl-cavitand body in acetone solution under −150 mV in a symmetrical aqueous solution of CaCl$_2$ (0.5 mol/L)

In order to test the dimer principle formation of artificial channel activity we performed several experiments in which the tetramethylcavitand was added to the lipid bilayer from only one side and not from both sides.

Figure 29: Patch clamp recording of tetramethyl-cavitand body in acetone solution added to the lipid bilayer from only one side (inner compartment), under −150 mV in a symmetrical aqueous solution of KCl (0.5 mol/L)
In all these cases, the patch clamp traces indicate no channel activity. The absence of any ion channel activity (Figure 29) is conform to the proposed dimerisation artificial channel mechanism. To form an artificial ion channel, the compounds should diffuse from both: measuring and reference compartments of the chamber into the bilayer. When the compound is added to the lipid bilayer from only one side, no ion channel is forming because the tetramethyl-cavitand compound do not flip-flop between the adjacent compartments of the lipid bilayer.

- **Calix[4]resorcinarene type with short alkyl chains**

The investigated calix[4]resorcinarene cavitand type has short alkyl tails (C₆H₁₃) attached to the lower rim (Figure 30).

![Calix[4]resorcinarene cavitand type with short alkyl chains at lower rim (C₆H₁₃)](image)

**Figure 30:** Calix[4]resorcinarene cavitand type with short alkyl chains at lower rim (C₆H₁₃)

The patch clamp experiments indicate no electrical current through the lipid bilayer regardless with the different concentrations and different applied potential. Figure 31 shows the patch clamp current recording for calix[4]resorcinarene cavitand type at -50 mV. The absence of ion channel activity in patch clamp traces shows that the short alkyl tails (C₆H₁₃ in Figure 31) are not capable of spanning half of the membrane.
thickness. The short alkyl chains attached to the lower rim of calix[4]resorcinarene cavitand could not interdigitate with the other short alkyl chains from other molecules and does not form ion channels.

**Figure 31:** Patch clamp recording for calix[4]resorcinarene cavitand type (C6H13) in acetone solution (10⁻⁸ mol/L) under –150 mV and 0.5 mol/L symmetric KCl electrolytic solution

Beside the tests carried out with electrolytic solution of KCl, the patch clamp experiments were performed using another different electrolytic solutions of NaCl, CsCl, CaCl2.

**Figure 32:** Patch clamp recording of calix[4]resorcinarene cavitand type (C6H13) in acetone solution, under –150 mV in a symmetrical aqueous solution of NaCl (0.5 mol/L)
Despite varied concentrations of calix[4]resorcinarene cavitand (C₆H₁₃) and applied potentials under different electrolytic solutions, no ion channel current traces were observed (e.g., patch clamp recording of calix[4]resorcinarene cavitand under NaCl solution in Figure 32).

As a next step we investigated calix[4]resorcinarene cavitand type with short alkyl tails (C₅H₁₁) at lower rim but with phosphatidylcholine groups attached to the upper rim (Figure 33). With this experiment we want to test the influence of phosphatidylcholine (PC) groups. These phosphatidylcholine groups are present also on the lipid (lecithin S 75) structure from which the bilayer is forming. These groups are the main components of the hydrophilic groups of the lipid (70 % phosphatidylcholine). Compare to the previous investigated compounds, such phosphatidylcholine groups on the upper rim allow calix[4]resorcinarenes to get a strong anchor of its hydrophilic part into the hydrophilic lipid bilayer part.

Figure 33: Calix[4]resorcinarene cavitand type with phosphatidylcholine groups and short alkyl chains at lower rim (C₅H₁₁)
Integration of artificial ion channels into lipid bilayer

The hydrophobic part of the calix[4]resorcinarene cavitand formed by the very short alkyl chains (C₅H₁₁) need also to integrate into the hydrophobic part of the lipid bilayer in order to exhibit ion channel activity.

Figure 34: Patch clamp recording for calix[4]resorcinarene cavitand type (C₅H₁₁) in acetone solution (10⁻⁸ mol/L) under –150 mV and 0.5 mol/L symmetric KCl electrolytic solution.

The patch clamp experiments indicate no electrical current (Figure 34) through the lipid bilayer regardless with the different concentrations, different type of ions (K⁺, Na⁺, Cs⁺) and different applied potential.

In conclusion, the channel recordings dependence of the length of the aliphatic chains of calix[4]resorcinarenes cavitand indicate that two molecules firstly must co-align across the membrane and form a dimer to allow the ion flux. If the aliphatic chains are long enough to span the whole length of the lipid bilayer, ion channel activity can be observed. When the aliphatic chains lengths are too short, and the ends cannot meet within the lipid bilayer to allow the formation of an ion channel, then no current can be observed.

Our patch clamp recordings indicate successfully channel activity in case of tetramethyl-cavitand type due to its long aliphatic tails (C₁₁H₂₃) attached to the lower
Integration of artificial ion channels into lipid bilayer

rim but indicate no channel activity in case of calix[4]resorcinare cavitand type because of its short aliphatic tails (C₆H₁₃) at the lower rim. The degree of alkyl tails interdigitation increase with the increasing tail length to afford a more ordered pore, which facilitates the ion permeation, cf. 53,77.

The patch clamp tests proved that tetramethyl-cavitand has contact with both: the hydrophilic and hydrophobic parts of the bilayer. The cavity formed by the four aromatic rings and extended by four alkyl chains exclude the entry of lipid molecules and provide the channel pore for the passage of ions. The appearance of the open states of the ion channels formed by tetramethyl-cavitand body is a stochastic process determined by chance and it cannot be controlled. At a certain time the exact switch of the channels cannot be predicted. The changes between open and closed conformations of the channels can be only recorded, but there could not be manipulated.

Moreover, tetramethyl-cavitand reveal ion channel activity that has K⁺ and Na⁺ selectivity indicated by representative ion channel current traces (Figure 25, respectively Figure 26). The patch clamp experiments provide evidence that tetramethyl-cavitand is not permissive for Cs⁺ and Ca²⁺ while no electrical current is measured through the lipid bilayer regardless variations in the applied electrical potentials and in the concentrations of the investigated compound (Figure 27, respectively Figure 28). Cs⁺ with a bigger ionic radius (ionic radius = 170 pm) compare with K⁺ (ionic radius = 138 pm) or Na⁺ (ionic radius = 102 pm) cannot pass through the channel formed by tetramethyl-cavitand body. Moreover, Ca²⁺ (ionic radius = 100 pm) cannot pass through the channel, despite its smaller size compared with K⁺ (ionic radius = 138 pm) or Na⁺ (ionic radius = 102 pm).

In case of K⁺ and Na⁺ selectivity, the observed conductance states remain approximate constant over all the current records. This is specific for ion channel activity and is in contrast with artificial molecular aggregate formation where were observed different conductance levels assigned to the action of different sized aggregates150.

On the same time, our selectivity experiments indicate artificial ion channel activity formation versus leaks through lipid bilayer. The pores formed by tetramethyl-cavitand through lipid bilayer are not only open holes of different diameters, since channels
allow the passage of certain ions (K$^+$ and Na$^+$) but do not allow the passage of larger (Cs$^+$) or even smaller ones (Ca$^{2+}$). This support the theory that there is a great energy expenditure required for dehydration, the necessary stage involved in the transport of cations through the ion channels$^{112}$.


Basic experiments to integrate the upper-rim substituted calix[4]resorcinarenes into a lipid bilayer were accomplished for multi-chromophore systems: thermodynamically EE-favoured conformation of 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand 7 (Figure 16) and for the thermodynamically EEEE-favoured conformation of tetrakis(p-phenylazophenylaminomethyl)-cavitand 5 (Figure 16). The basic experiments were accomplished for the multi-chromophore system because these compounds exhibit the highest probability to obtain a full external control of the light gating mechanism. In case of the single-chromophore system 11,17,23-trimethyl-(5-p-phenylazophenylaminomethyl)-cavitand 8 we did not performed the patch clamp experiments because a similar system with one azobenzene molecule constructed on the top of a natural ion channel (gramicidin A) was already investigated$^{151}$. The reported results show that such systems with one azobenzene group significant affect the blocking of natural ion channel, but do not achieve the complete blockage of the ion channel. Additional azobenzene groups at the rim of the cavity (like in our multi-chromophore systems; 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand 7, respectively tetrakis(p-phenylazophenylaminomethyl)-cavitand 5) increase the probability to close the artificial ion channel completely. Both multi-chromophore compounds were successfully integrated into lipid bilayer and exhibit short open and closed states of the channels measured via patch clamp system.

For 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand 7, the thermodynamically EE-favoured conformation was tested using KCl, CsCl and CaCl$_2$ electrolytic solutions. Independent measurements were performed for each type of electrolytic solution.
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Figure 35: Patch clamp recording of the thermodynamically favoured EE conformation of 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand 7 in acetone solution at 150 mV in a symmetric solution of KCl (0.5 mol/L)

Basic patch clamp recordings for 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand 7 using KCl solutions provide successfully integration into lipid bilayer with short opening and closing states (Figure 35).

The selectivity of the thermodynamically favoured EE conformations of 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand 7 was monitored via patch clamp system with additional measurements using CsCl and CaCl₂ electrolytic solutions.

Figure 36: Patch clamp recording of the thermodynamically favoured EE conformation of 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand 7 in acetone solution at 150 mV, in a symmetric solution of CsCl (0.5 mol/L)
In case of both type of measurements with Cs$^+$ (Figure 36) and Ca$^{2+}$ (Figure 37) we observed no electrical current measured through the lipid bilayer regardless reasonable variations in the applied potential and different concentrations of 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand 7.

In conclusion, our basic patch clamp experiments show evidence that 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand 7 integrate successfully into the lipid bilayer, providing the channel pore for the passage of ions. Moreover, the basic experiments in case of 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand 7, indicate artificial ion channel activity formation versus leaks through lipid bilayer. The pores formed by thermodynamically favoured EE conformations of 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand 7 allow the passage of certain ions like K$^+$ but do not allow passage of larger Cs$^+$ or even smaller ones Ca$^{2+}$ through the lipid bilayer.

In case of the tetrakis(p-phenylazophenylaminomethyl)-cavitand 5 it was tested the thermodynamically EEEE-favoured conformation (Figure 38) in independent experiments using KCl, CsCl, CaCl$_2$ electrolytic solutions.
For each three types of electrolytic solutions it was performed five separate experimental runs.

**Figure 38:** Patch clamp recording of the thermodynamically EEEE-favoured conformation of tetrakis(p-phenylazophenylaminomethyl)-cavitand 5 under \(-150\) mV in a symmetrical aqueous solution of KCl (0.5 mol/L).

The tetrakis(p-phenylazophenylaminomethyl)-cavitand 5 diffused from both sides into the bilayer and a stable ion channel with rectangular current patterns is observed under KCl electrolytic solution (Figure 38).

In case of thermodynamically trans favoured conformation of tetrakis(p-phenylazophenylaminomethyl)-cavitand 5, the experiments that indicate channel activity in KCl electrolytic solution were completed with independent patch clamp experiments in symmetrical CsCl and CaCl₂ solutions.
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**Figure 39:** Patch clamp recording of the thermodynamically trans favoured conformation of tetrakis(p-phenylazophenylaminomethyl)-cavitand 5 under –150 mV in electrolytic CsCl aqueous solution (0.5 mol/L)

**Figure 40:** Patch clamp recording of the thermodynamically trans favoured conformation of tetrakis(p-phenylazophenylaminomethyl)-cavitand 5 under –150 mV in electrolytic CaCl$_2$ aqueous solution (0.5 mol/L)

In all experiments, the patch clamp measurements of tetrakis(p-phenylazophenylaminomethyl)-cavitand 5 indicate no selectivity for Cs$^+$ (Figure 39), since no electrical current could be measured through the lipid bilayer. In case of Ca$^{2+}$, all experiments
indicate no ion translocation through the tetrakis(p-phenylazophenylaminomethyl)-cavitand 5 (Figure 40).

Moreover, for 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand 7 and tetrakis(p-phenylazophenylaminomethyl)-cavitand 5 it was monitored also the channel activity when the compounds were added to the lipid bilayer from only one side. During all these tests it was recorded no electrical current. This proves that both compounds: tetrakis(p-phenylazophenylaminomethyl)-cavitand 5 and 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand 7 do not exhibit a flip-flop effect between the adjacent compartments of the lipid bilayer to allow the dimerisation and the ion channel formation.

In conclusion, the basic patch clamp test of the thermodynamically favoured EE conformation of 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand 7 and of the thermodynamically favoured EEEE conformation of tetrakis(p-phenylazophenylaminomethyl)-cavitand 5 proved that both compounds incorporate successfully into lipid bilayer under KCl electrolytic solution and form ion channel with one conductance level. The patch clamp recordings showed frequent and very fast transitions between the open and closed states of the artificial channel with stable current level. The very short opening states of artificial ion channels made by tetrakis(p-phenylazophenylaminomethyl)-cavitand 5 and respective by 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand 7 are comparable with the natural ion channels: e.g., Shaker channel (Figure 41)\(^{152}\), acetylcholine channel (ACh) (Figure 42)\(^{153}\).

![Figure 41: Patch clamp activity of natural Shaker ion channel\(^{152}\)](image)
Integration of artificial ion channels into lipid bilayer

**Figure 42:** Ion channel recording from acetylcholine channel (ACh) with open and closed levels. The fast jumps between the open conformations and the closed conformations of the ion channel activities recorded from tetrakis(p-phenylazophenylaminomethyl)-cavitand 5 and from 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand 7 are similar not only to the natural ion channels types but also to many artificial ion channels types, e.g., cyclic octa-peptides artificial ion channels or beta-tetrapeptide artificial ion channels which exhibit very short opening states.

The pores formed by thermodynamically favoured EE conformations of tetrakis(p-phenylazophenylaminomethyl)-cavitand 5 similar with the 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand 7 and the tetramethyl-cavitand body, allow the passage of certain ion (K⁺) but do not allow the passage of larger (Cs⁺) or smaller ones (Ca²⁺). These basic experiments indicate ion channel formations which are in contrast with the leaks formation governed by the principle: the smaller the size of the ions, the easier to go through the pore. Our patch clamp measurements constitute relevant and promising steps in further ion channel activity investigations of our developed light-gated artificial ion channels.
8 SUMMARY

In the first chapter, the key topic of this dissertation, namely the engineering of a rigid and reversible gate for artificial ion channels made from calix[4]resorcinarene is introduced. The goal of this project is the development of artificial channels that can be actuated by light and thus controlled efficiently. The selected approach is based on fast, light-driven processes that use steric differences between trans and cis isomers. Light-gated artificial ion channels are aimed at eliminating of the stochastic mechanism of artificial ion channels. Such a reversible photocontrol should be a powerful tool for using artificial ion channels as the basis for the development of new pharmaceuticals and drug delivery systems, as photoswitches, and in the field of microfluidics.

In the second chapter, a review of artificial and natural ion channels as well as their main limitations in technical environmental is given. The mechanism by which gramicidin A, the most studied natural ion channel model for artificial ion channels functions is presented. The body of our light-operated artificial channels was based on calix[4]resorcinarene molecules that were selected because they provide a well-defined cavity that offers simultaneous polar and non-polar features for artificial ion channel formation. This type of molecule builds ion channels by forming tail-to-tail associated dimers in the same manner as gramicidin A.

The following chapters describe the lipid membrane-polymer system and the relationship between artificial ion channels and lipid bilayers. The transport of ions through the lipid bilayer can be accomplished by ion channels. The patch clamp system is described; this device is the key technique for studying ion channel activity. Other instruments and chemicals used to achieve the main goal are also presented.

In the next chapter, the synthesis and characterisation of the upper-rim azobenzene substituted calix[4]resorcinarenes are described. These compounds are based on
different numbers of azobenzene groups attached to the upper-rim: tetrakis(p-phenylazo-phenylaminomethyl)-cavitand (with four azobenzene groups), 23-methyl-tris(5,11,17- p-phenylazophenylaminomethyl)-cavitand (with three azobenzene groups), 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand (with two azobenzene groups) and 11,17,23-trimethyl-(5-p–phenylazophenylaminomethyl)-cavitand (with one azobenzene group).

The sixth chapter focuses on the isomerisation experiments of the isolated gate part of the light-operated artificial ion channels and on the results of the photo responsive behaviour of single and multiple azobenzene substituted systems under irradiation. Such photochromic compounds were interconverted photochemically using two different optical filters, a band-pass filter with $295 \text{ nm} < \lambda < 400 \text{ nm}$ for the trans-cis transformation and one filter with $\lambda > 475 \text{ nm}$ for the back reaction. The conformational effects of photoisomerisation in these systems are discussed.

In chapter seven, patch clamp studies of two types of light-gated artificial ion channels are described, namely the thermodynamically EEEE-favoured conformation of tetrakis(p-phenylazophenylaminomethyl)-cavitand and the thermodynamically EE-favoured conformation of 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand. Basic patch clamp experiments proved that these compounds successfully integrate into the lipid bilayer and exhibit channel activity with one conductance level. Independent patch clamp selectivity measurements of the thermodynamically EEEE-favoured conformation of tetrakis(p-phenylazophenylaminomethyl)-cavitand and of the thermodynamically EE-favoured conformation of 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand are also described. The patch clamp experiments performed with different ions demonstrated selectivity for $\text{K}^+$ but not for $\text{Cs}^+$ and $\text{Ca}^{2+}$. These basic results indicate that the thermodynamically favoured E conformations of tetrakis(p-phenylazophenylaminomethyl)-cavitand and the thermodynamically EE-favoured conformations of 11,23-dimethyl-bis(5,17-p-phenylazophenylaminomethyl)-cavitand exhibit channel activity and do not produce leaks into membrane since these channels allow the passage of only certain ions.
In conclusion, for the first time, light gated artificial ion channels with azobenzene groups on the upper rim have been synthesised and characterised. Such photochromic systems are reversible, switchable and exhibit photo-responsive conformational effects under different irradiation conditions. Basic experiments for the integration of these systems into lipid bilayers were performed for two light-gated artificial ion channels. These results provide strong evidence for successful ion channel formation and a leak-free bilayer integration.
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Versicherung

Hiermit versichere ich, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht. Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Dresden, 09. November 2006

Erklärung

Die vorliegende Dissertation wurde unter der wissenschaftlichen Betreuung von Prof. Reiner Salzer an der TU-Dresden im Institut für Analytische Chemie angefertigt.
Frühere Promotionsverfahren haben nicht stattgefunden.

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