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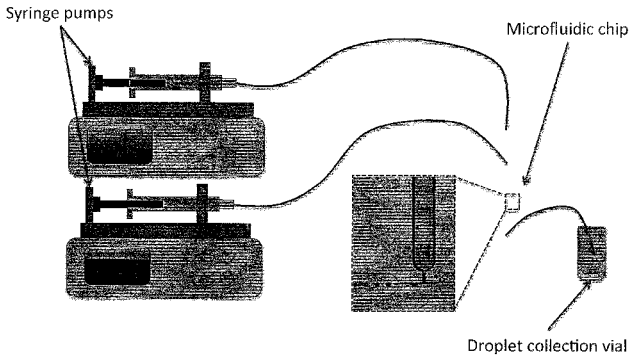

Fig. 1

(57) **Abstract**: The invention provides a method of forming a membrane between a first volume of polar medium and a second volume of polar medium, which method comprises: (a) providing a first volume comprising polar medium and a second volume comprising polar medium which are separated from one another by an apolar medium, wherein at least one of said first and second volumes comprises a layer comprising amphipathic molecules, at the interface between the polar medium and the apolar medium, wherein each of the amphipathic molecules comprises a first outer hydrophilic group, a hydrophobic core group, and a second outer hydrophilic group, wherein each of the first and second outer hydrophilic groups is linked to the hydrophobic core group; and (b) causing the first and second volumes to come into contact with one another to form a membrane comprising said amphipathic molecules between the first and second volumes. The invention also provides a system comprising a membrane between a first volume of a polar medium; and a second volume of a polar medium, which membrane comprises the amphipathic molecules, and wherein the first volume of polar medium is within an apolar medium.

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DROPLET INTERFACES

Field Of The Invention

The invention relates to a membrane between first and second volumes of a polar medium. The invention also relates to a method of forming such a membrane.

Background To The Invention

Lipid bilayers are thin polar membranes formed from two layers of lipid molecules. Lipid bilayers are found in cell membranes of most living organisms and are usually composed of phospholipids. They are impermeable to most hydrophilic molecules and ions, and enable cells to regulate their salt concentrations and pH by pumping ions across the lipid bilayer using transmembrane proteins known as ion pumps. Lipid bilayers, or more generally bilayers of amphipathic molecules, also serve as excellent platforms for a range of experimental studies. Holden *et al*, J. Am. Chem. Soc. 2007, 129, 8650-8655 disclose the formation of functional bionetworks of aqueous droplets comprising lipid bilayers provided between droplets. Such networks can act as light sensors, batteries and electrical components by incorporating pumps, channels and pores into the bilayers. Sackmann, Science, New Series, Vol 271, No.5245 (Jan 5, 1996), pp. 43-48 provides a review of the scientific and practical applications of supported lipid-protein bilayers including their use in electrooptical biosensors. Jung *et al*, J. Am. Chem. Soc., 2009, 131 (3), 1006-1014 have developed optical assays for the detection of protein ligand binding on supported bilayers. The provision of ion channels in highly resistive amphipathic lipid bilayers for the detection of DNA and other analytes is well documented, see for example Bayley *et al*, Nature, Vol 413, September 2001. Aqueous solutions are provided on either side of the lipid bilayer and ion flow through the nanopore takes place under a potential gradient. DNA is caused to translocate the ion channel and the change in ion flow during translocation of DNA through the channel is measured. Due to the high resistance of the lipid bilayer, ion flow takes place exclusively through the ion channel. The lipid bilayer may be suspended across an aperture of a substrate and formed by methods well known in the art such as patch clamping or painting.

WO2009/077734 discloses a plurality of individually addressable lipid bilayers formed across an array of microwell apertures, each microwell containing an electrode and an aqueous medium in contact with the lipid bilayer.

WO2009/012552 discloses a bilayer of amphipathic lipid molecules formed between two droplets comprising a layer of amphipathic molecules containing a hydrophilic medium,

the droplets being provided in a hydrophobic medium. Ion flow across the lipid bilayer is measured with electrodes provided within the hydrophilic interior of each droplet.

An amphipathic molecule may be considered as comprising a polar hydrophilic region attached to a non-polar hydrophobic region. A bilayer may be formed from two monolayers of amphiphilic molecules, wherein in aqueous solution, the polar groups face towards the hydrophilic media on either side of the bilayer and the hydrophobic groups face inwards.

WO2009/024775 discloses a method for producing a droplet interface bilayer (DIB) wherein droplets are prepared by contacting an oil/lipid solution with an aqueous solution and the resulting droplets are brought into contact with an aqueous agarose gel support layer.

Phospholipids such as 1,2-diphytanoyl-sn-glycero-3-phosphatidylcoline (DPhPC) are routinely used to form lipid bilayers. However drawbacks that are sometimes associated with lipid bilayers include that they are not particularly robust and are prone to rupture, for example by digestion by enzymes, and are not able to withstand large potential differences.

US6,723,814 discloses a planar membrane formed from amphiphilic copolymers having hydrophilic and hydrophobic segments. The copolymer may be an ABA triblock having methyloxazoline hydrophilic segments and a dimethylsiloxane hydrophobic core (PMOXA-PDMS-PMOXA). Membranes formed from this triblock are able to withstand higher potential differences than lipid membranes (Table 1 of US6,723,814).

US6,916,488 describes the preparation of vesicles made from PMOXA-PDMS-PMOXA in a hydrophilic medium (type ABA). The structure of an amphipathic ABA triblock vesicle (a droplet in a hydrophilic medium having a hydrophilic interior) may be conceptualised as a monolayer of triblock polymer in which the polymer molecules have a linear configuration in which the two hydrophilic 'A' segments face the respective hydrophilic solutions on either side of the vesicle wall. Such a configuration, which is shown in Figure 1 of US6,916,488, would not however seem suitable for stabilising aqueous droplets in oil. Such ABA molecules do not therefore seem to be a viable alternative to the lipids described in WO2009/024775, for producing a droplet interface layer from a water-in-oil system.

There is thus an ongoing need to provide alternative methods for producing interface membranes that provide improved stability compared to conventional lipid bilayers.

Summary Of The Invention

It is a finding of the invention that contacting a polar medium with an apolar medium containing ABA molecules results in spontaneous formation of a layer of the ABA molecules

around the polar medium, at the apolar-polar interface. Moreover, when two such volumes of polar media are then brought together, through the apolar medium, a stable membrane of ABA molecules forms at the interface between the first and second polar volumes.

The resultant membrane, being synthetic, has been shown to be robust, stable and less susceptible to degradation from detergents and proteins than conventional lipid systems.The membrane is also able to withstand larger potential differences applied across it. Proteins, such as transmembrane protein pores, may be inserted into the membrane and used to characterise target analytes, including DNA.

The successful formation of stable ABA membranes in this manner is counter-intuitive; the fact that the ABA molecules spontaneously form a layer at the polar-apolar interfaces, and then subsequently produce a stable membrane between two polar phases, was unexpected.

Accordingly, the invention provides in a first aspect a method of forming a membrane between a first volume of polar medium and a second volume of polar medium, which method comprises:

(a) providing a first volume comprising polar medium and a second volume comprising polar medium which are separated from one another by an apolar medium,

wherein at least one of said first and second volumes comprises a layer comprising amphipathic molecules, at the interface between the polar medium and the apolar medium,

wherein each of the amphipathic molecules comprises a first outer hydrophilic group, a hydrophobic core group, and a second outer hydrophilic group, wherein each of the first and second outer hydrophilic groups is linked to the hydrophobic core group; and

(b) causing the first and second volumes to come into contact with one another to form a membrane comprising said amphipathic molecules between the first and second volumes of polar medium.

The first volume may be provided within the apolar medium.

In another aspect, the invention provides a membrane which is obtainable by the method of the invention.

In another aspect, the invention provides a system comprising

a first volume of a polar medium;

a second volume of a polar medium; and

a membrane between the first and second volumes of polar medium, which membrane comprises amphipathic molecules,

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wherein each of the amphipathic molecules comprises a first outer hydrophilic group, a hydrophobic core group, and a second outer hydrophilic group, wherein each of the first and second outer hydrophilic groups is linked to the hydrophobic core group,

and wherein the first volume is within an apolar medium.

The system may further comprise a layer of said amphipathic molecules at an interface between the first volume of polar medium and the apolar medium.

The system may comprise a plurality of first volumes within the apolar medium and a plurality of respective membranes between the plurality of first volumes and the second volume.

The system may comprise a plurality of first volumes within the apolar medium, a plurality of second volumes, and a plurality of membranes provided between the respective first and second volumes. The one or more second volumes may also be provided within the apolar medium.

The invention also provides a volume comprising polar medium, which volume is disposed within an apolar medium, and which volume has a layer comprising amphipathic molecules around a surface thereof, between the polar medium and the apolar medium, wherein each of the amphipathic molecules comprises a first outer hydrophilic group, a hydrophobic core group, and a second outer hydrophilic group, wherein each of the first and second outer hydrophilic groups is linked to the hydrophobic core group, and wherein each of the amphipathic molecules is a copolymer comprising at least three polymer segments, wherein the hydrophobic core group is an inner hydrophobic polymer segment, B, and the first and second outer hydrophilic groups are first and second outer hydrophilic polymer segments, A_1 and A_2.

Further provided is a process for producing a volume comprising polar medium, which volume is disposed within an apolar medium, and which volume has a layer comprising amphipathic molecules around a surface thereof, between the polar medium and the apolar medium, wherein each of the amphipathic molecules comprises a first outer hydrophilic group, a hydrophobic core group, and a second outer hydrophilic group, wherein each of the first and second outer hydrophilic groups is linked to the hydrophobic core group, and wherein each of the amphipathic molecules is a copolymer comprising at least three polymer segments, wherein the hydrophobic core group is an inner hydrophobic

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polymer segment, B, and the first and second outer hydrophilic groups are first and second hydrophilic polymer segments, A_1 and A_2,

which process comprises:

(i) introducing a volume of a polar medium into an apolar medium;

(ii) providing the amphipathic molecules, in the apolar medium or the polar medium or both, either before or after (i); and

(iii) leaving the volume of polar medium for a time sufficient for the layer comprising the amphipathic molecules to form at the interface between the polar medium and the apolar medium.

The membrane may comprise a transmembrane pore for the determination of the presence of an analyte in or movement of an analyte through the pore. The presence and/or amount of a transmembrane pore in the membrane may also be determined.

Accordingly, the invention further provides a method of characterising a target analyte, comprising:

(a) contacting the target analyte with a transmembrane pore present in a membrane of the system of the invention as defined herein,

(b) taking one or more measurements as the analyte moves with respect to the pore or of the presence of analyte within the pore, wherein the measurements are indicative of one or more characteristics of the target analyte and thereby characterising the target analyte.

Further provided is a method of forming a sensor for characterising a target polynucleotide, comprising forming a complex between (a) a pore present in a membrane of the system of the invention as defined herein, and (b) a polynucleotide binding protein and thereby forming a sensor for characterising the target polynucleotide.

The invention also provides a sensor for characterising a target polynucleotide, comprising a complex between (a) a pore present in a membrane of the system of the invention as defined herein, and (b) a polynucleotide binding protein, and thereby forming a sensor for characterising the target polynucleotide.

Additionally provided is a kit for characterising a target polynucleotide comprising (a) a pore present a membrane of the system of the invention as defined herein, and (b) a polynucleotide binding protein and thereby forming a sensor for characterising the target polynucleotide.

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In another aspect, the invention provides an apparatus for characterising target polynucleotides in a sample, comprising (a) a plurality of pores present in a plurality of membranes of one or more systems of the invention as defined herein, and (b) a plurality of polynucleotide binding proteins.

The polar medium may be a hydrophilic medium. The apolar medium may be a hydrophobic medium.

Brief Description Of The Figures

Fig. 1 shows a schematic for the droplet generation setup. This setup consists of two syringe pumps (Elite, Harvard Apparatus), two gastight syringes (Hamilton), Peak tubing (Upchurch Scientific), and a custom made T-junction microfluidic chip.

Fig. 2 shows droplet stability experiments. A) shows the stability of the 6-33-6 Polymersource droplets in AR20 oil changed over time. After 20 hours these droplets were found to have not merged. B) shows examples of unstable, meta-stable and stable droplets for illustrative purposes.

Fig. 3 shows the experimental set-up for the droplet-interface-bilayer experiments.

Fig. 4 shows how the droplet-interface-bilayer experiment is set-up inside the faraday cage. A) shows a schematic view and B) shows the droplets as viewed from the microscope below the faraday cage.

Fig. 5 shows an example electrical trace illustrating how the capacitance of two 6-33-6 PolymerSource droplets in AR20 oil increased over time.

Fig. 6 shows an example electrical trace illustrating how a sharp current increase was observed when MspA-(B2C) (SEQ ID NO: 25, which is a variant of SEQ ID NO: 2 with the following mutations G75S/G77S/L88N/Q126R) inserted into 6-33-6 Polymersource tri-block co-polymer droplets in AR20 oil. Instances where pores have inserted into the tri-block are indicated by black arrows.

Fig. 7 shows in section A) an example electrical trace illustrating how the capacitance of two 6-45PE-6 PolymerSource droplets in hexadecane increased over time and in section B) how an example electrical trace illustrating how a sharp current increase was observed when MspA-(B2C) (SEQ ID NO: 25, which is a variant of SEQ ID NO: 2 with the following mutations G75S/G77S/L88N/Q126R) inserted into 6-45PE-6 Polymersource tri-block co-polymer droplets in AR20 oil. Instances where pores have inserted into the tri-block are indicated by black arrows.

Description Of The Sequence Listing

SEQ ID NO: 1 shows the codon optimised polynucleotide sequence encoding the MS-B1 mutant MspA monomer. This mutant lacks the signal sequence and includes the following mutations: D90N, D91N, D93N, D118R, D134R and E139K.

SEQ ID NO: 2 shows the amino acid sequence of the mature form of the MS-B1 mutant of the MspA monomer. This mutant lacks the signal sequence and includes the following mutations: D90N, D91N, D93N, D118R, D134R and E139K.

SEQ ID NO: 3 shows the polynucleotide sequence encoding one monomer of α-hemolysin-E111N/K147N (α-HL-NN; Stoddart *et al.*, PNAS, 2009; 106(19): 7702-7707).

SEQ ID NO: 4 shows the amino acid sequence of one monomer of α-HL-NN.

SEQ ID NOs: 5 to 7 show the amino acid sequences of MspB, C and D.

SEQ ID NO: 8 shows the polynucleotide sequence encoding the Phi29 DNA polymerase.

SEQ ID NO: 9 shows the amino acid sequence of the Phi29 DNA polymerase.

SEQ ID NO: 10 shows the codon optimised polynucleotide sequence derived from the *sbcB* gene from *E. coli*. It encodes the exonuclease I enzyme (EcoExo I) from *E. coli*.

SEQ ID NO: 11 shows the amino acid sequence of exonuclease I enzyme (EcoExo I) from *E. coli*.

SEQ ID NO: 12 shows the codon optimised polynucleotide sequence derived from the *xthA* gene from *E. coli*. It encodes the exonuclease III enzyme from *E. coli*.

SEQ ID NO: 13 shows the amino acid sequence of the exonuclease III enzyme from *E. coli*. This enzyme performs distributive digestion of 5' monophosphate nucleosides from one strand of double stranded DNA (dsDNA) in a 3' – 5' direction. Enzyme initiation on a strand requires a 5' overhang of approximately 4 nucleotides.

SEQ ID NO: 14 shows the codon optimised polynucleotide sequence derived from the *recJ* gene from *T. thermophilus*. It encodes the RecJ enzyme from *T. thermophilus* (*Tth*RecJ-cd).

SEQ ID NO: 15 shows the amino acid sequence of the RecJ enzyme from *T. thermophilus* (*Tth*RecJ-cd). This enzyme performs processive digestion of 5' monophosphate nucleosides from ssDNA in a 5' – 3' direction. Enzyme initiation on a strand requires at least 4 nucleotides.

SEQ ID NO: 16 shows the codon optimised polynucleotide sequence derived from the bacteriophage lambda *exo* (*redX*) gene. It encodes the bacteriophage lambda exonuclease.

SEQ ID NO: 17 shows the amino acid sequence of the bacteriophage lambda exonuclease. The sequence is one of three identical subunits that assemble into a trimer. The enzyme performs highly processive digestion of nucleotides from one strand of dsDNA, in a 5'-3'direction (http://www.neb.com/nebecomm/products/productM0262.asp). Enzyme

initiation on a strand preferentially requires a 5' overhang of approximately 4 nucleotides with a 5' phosphate.

SEQ ID NO: 18 shows the amino acid sequence of Hel308 Mbu.

SEQ ID NO: 19 shows the Hel308 motif of Hel308 Csy.

SEQ ID NO: 20 shows the amino acid sequence of Hel308 Tga.

SEQ ID NO: 21 shows the amino acid sequence of Hel308 Mhu.

SEQ ID NO: 22 shows the amino acid sequence of TraI Eco.

SEQ ID NO: 23 shows the amino acid sequence of XPD Mbu.

SEQ ID NO: 24 shows the polynucleotide sequence encoding MspA-(B2C) (a variant of SEQ ID NO: 2 with the following mutations: G75S/G77S/L88N/Q126R).

SEQ ID NO: 25 shows the amino acid sequence of MspA-(B2C) (a variant of SEQ ID NO: 2 with the following mutations: G75S/G77S/L88N/Q126R).

Detailed Description Of The Invention

The method of the first aspect of the invention is straightforward to perform and results in a robust membrane comprising the amphipathic molecules, which can be used in a wide range of studies and applications in the field of biotechnology. The membrane is less susceptible to degradation than a conventional phospholipid bilayer, and is also able to withstand larger potential differences. The membrane has be shown to be more robust and stable having a longer lifetime than a convention lipid bilayer membrane enabling sensors to be provided and stored with prefabricated membranes. It also allows detergent and protein containing samples such as biological samples to be directly applied to the membrane for the detremination of an analyte.

The step of providing the first and second volumes comprising polar medium which are separated from one another by an apolar medium, may be performed very easily. It usually comprises contacting each of the volumes comprising polar medium with an apolar medium.

The polar medium may be provided in the form of one or more droplets and/or one or more beads. Droplets may be formed, for instance by introducing polar medium into the apolar medium by syringe or pipette. Droplet or droplets of polar medium can also be formed in an apolar medium using a microfluidic device, for instance as described in Example 1 hereinbelow. The sizes of the channels within the microfluidic device, and the flow rates of the apolar and polar media through the microfluidic device, can be varied as desired to

control the size of the polar droplets produced. Particularly small droplets can be produced by using a microfluidic device, for instance in the size (diameter) range of from 5 μm to 500 μm. Droplets of polar medium formed within a microfluidic device may be transferred into a bulk apolar medium, outside of the microfluidic device, if desired, for further manipulation. A bead or beads of polar medium may be formed within an apolar medium in a similar manner to droplets. For instance, a polar flowable medium which is capable of forming a bead, such as a hydrogel, can be introduced into the apolar medium by pipette or syringe.

Alternatively one or more pre-formed beads comprising polar medium may simply be dispensed into the apolar medium. Examples of such are a non-crosslinked or crosslinked hydrogel such as agarose or sepharose, or porous glass or plastic beads containing a polar medium. A bead may be formed *in-situ* from a droplet for example by cooling or crosslinking with UV. A bead introduced into the apolar medium may form a droplet, for example by melting.

As an alternative to providing the second volume within the apolar medium, the second volume may be applied to the surface of the apolar medium. This can be done by any suitable method. For instance, the polar medium can be applied to the surface of the apolar medium by pipette or syringe, or by using a flow cell. In another method, a volume of polar medium may be initially provided, for example in a vessel, and the apolar medium applied to the surface of the polar medium. The first volume of polar medium may be subsequently applied to the surface of the apolar medium in order to provide the interface between the two volumes of polar medium.

A plurality of membranes may be provided at the interfaces between a plurality of discrete volumes of polar medium and a layer of polar medium. The volumes of polar medium may be separated from each other by the apolar medium.

The amphipathic molecules may be provided in either the apolar or polar medium. In the case of providing a single volume of the polar medium in the apolar medium, the amphipathic molecules may be provided either before or after the apolar and polar media have been brought into contact with each other. In the case however where a plurality of volumes of polar media are provided in the apolar medium, for example in the form of an emulsion, the amphipathic molecules are preferably provided in either the apolar or polar medium prior to contacting the apolar and polar media to avoid merging of the volumes of polar media. After the amphipathic molecules have been provided and the apolar and polar media have been contacted with one another, a layer comprising the amphipathic molecules forms naturally, at the interface between the apolar medium and the polar medium. The rate

of formation of the layer of amphipathic molecules depends upon experimental factors such as the concentration of the amphipathic molecules present and whether they are provided within the apolar volume or within a polar volume. The time taken to form the amphipathic layer may vary and may be of the order of a few minutes or longer. The amphipathic molecules can be provided in the apolar or polar medium by dissolving them in the medium, or for instance by forming vesicles of the amphipathic molecules in the apolar or polar medium. The amphipathic molecules are usually provided in the apolar medium. Typically, they are dissolved in the apolar medium.

Without wishing to be bound by theory, it is thought that the amphipathic molecules in the or each layer at the interface between the polar medium and the apolar medium are probably folded, such that the hydrophobic core group faces outwards, away from the polar medium and towards the apolar medium, and such that the first and second outer hydrophilic groups face inwards, towards the polar medium. Thus, it may be the case that the or each layer of amphiphilic molecules at the interface between the polar medium and the apolar medium comprises a monolayer of the amphipathic molecules which are folded in that way. In cases where the molecule is a triblock ABA type copolymer, wherein each A is an outer hydrophilic polymer segment and B is an inner hydrophobic segment, the molecules in the layer may be U-shaped, such that the hydrophobic B group faces outwards, away from the polar medium and towards the apolar medium, and the two hydrophilic A groups face inwards, towards the polar medium.

The word "causing" as used in step (b) of the first aspect of the invention is intended to encompass, on the one hand, actively bringing the two volumes of polar medium into contact with one another, and, on the other hand, allowing the first and second volumes of polar medium to come into contact by themselves, i.e. allowing the two volumes of polar medium to contact one another and form the membrane by self-assembly.

The volumes of polar medium may be handled by a variety of techniques. For instance, a droplet or bead of polar medium may be moved by disposing an anchor having a hydrophilic outer surface inside the droplet or bead. Movement of the anchor allows the droplet or bead to be moved, for example to bring it into contact with another volume of polar medium. Such manipulation is described in Example 2 below, and in Figures 3 and 4, in which two electrodes having hydrophilic, agarose-coated contacts serve as the anchors.

In the step of causing the first and second volumes of polar medium to come into contact, the first and second volumes of polar medium move relatively towards each other

through the intervening apolar medium, and intervening apolar medium is displaced from between the two volumes.

As soon as the apolar medium has been sufficiently displaced from between the first and second volumes comprising polar medium, such that the first and second volumes contact each other, the membrane of the amphipathic molecules forms between the first and second volumes.

The time taken to form the membrane may vary between seconds to hours depending upon the experimental conditions. The formation of the membrane of amphipathic molecules between two volumes of polar medium can be measured experimentally, by monitoring the change in capacitance between the two volumes of polar medium. Such an experiment is described in Example 2 hereinbelow, in section 2.3. The results are shown in Figure 5, where the increase in capacitance over time demonstrated the formation of a membrane of the amphiphilic molecules between the two droplets of aqueous buffer that were tested. Thus, by monitoring capacitance, the skilled person can verify formation of the membrane comprising the amphipathic molecules between the first and second volumes of polar medium, in accordance with the method of the present invention.

Without wishing to be bound by theory, it is thought that the membrane formed between the two volumes of polar medium, in the process of the invention, may comprise a monolayer of the amphipathic molecules. In particular, it is thought that the membrane may comprise a monolayer of amphipathic molecules aligned next to one another such that the hydrophobic core groups are aligned to form a middle hydrophobic layer which is not in contact with either of the two volumes of polar medium, and such that the first and second outer hydrophilic groups are aligned to form first and second outer hydrophilic layers which contact the two volumes of polar medium on either side of the membrane.

If that is the case, and if, as postulated hereinbefore, the amphipathic molecules at the interfaces between the polar medium and the apolar medium are folded such that all the hydrophobic core groups face towards the apolar medium and all the first and second outer hydrophilic groups face towards the polar medium, then it is likely that the formation of the membrane in accordance with the method of the invention involves a re-arrangement of the amphipathic molecules, comprising unfolding of the amphipathic molecules.

Another possibility, however, is that the amphipathic molecules remain folded when the method of the invention is performed, and the membrane formed between the two volumes of polar medium comprises a bilayer of the folded amphipathic molecules, in which all the hydrophobic core groups face inwards towards the middle of the bilayer, and all the

outer hydrophilic groups face outwards, towards the first and second polar medium. Such a bilayer might for instance be formed by bringing two monolayers of folded amphipathic molecules together, by performing the method of the invention as defined hereinbefore in which each of the first and second volumes of polar medium comprises a layer comprising the amphipathic molecules at the interface between the apolar and polar medium.

The term bilayer as used herein refers to a membrane comprising two monolayers of amphipathic molecules. The term monolayer refers to a membrane formed from a single layer of amphipathic molecules.

The term "bead" typically refers to a volume of a medium which has a defined shape and is generally pre-formed. Examples of such are a glass or plastic porous bead containing polar medium, or an uncrosslinked or crosslinked hydrogel such as agarose or sepharose.

A droplet, on the other hand, refers to a volume of a flowable medium which typically does not have a preformed shape prior to insertion into the apolar medium. Examples of such are an aqueous solution or a hydrogel. The hydrogel may be heated prior to insertion in the apolar medium to increase its flowability. A bead may be formed *in situ* from a droplet in the apolar medium, for example by cooling or by crosslinking with UV. A bead added to the apolar medium may subsequently form a droplet, for example by melting.

The bead may have any particular shape such as spherical, rod, triangular, square, hexagonal or irregular.

More than two volumes comprising polar medium may be brought together to form a chain or network of such volumes, wherein each volume comprising polar medium contacts a neighbouring volume comprising polar medium. Ion channels may be provided between the respective volumes to provide an interconnected ionic network.

In a preferred embodiment of the method of the invention, the second volume comprising polar medium is provided on the surface of the apolar medium. The second volume may be a sample suspected of comprising a target analyte of interest and measurements can be made to characterise the analyte. The second volume may be a sample comprising a target analyte.

The target analyte may for instance be a metal ion, an inorganic salt, a polymer, an amino acid, a peptide, a polypeptide, a protein, a nucleotide, an oligonucleotide, a polynucleotide, a dye, a bleach, a pharmaceutical, a diagnostic agent, a recreational drug, an explosive or an environmental pollutant. The protein may be a transmembrane protein. In particular, the target analyte is a target polynucleotide. The sample may for instance be a biological sample.

In some embodiments of the method of the invention, the first volume is a droplet or bead and the second volume is a sample comprising or suspected of comprising a target analyte.

The mean diameter of the droplets or beads is typically from about 5 μm to about 500 μm.

The or each layer comprising the amphipathic molecules as well as the resultant membrane or membranes formed between the volumes of polar medium may additionally comprise further molecules.

The further molecules may include functional molecules, such as transmembrane pores and membrane proteins, which will be described in further detail hereinbelow. Additionally or alternatively, the further molecules may include additional amphiphilic molecules, i.e. amphiphilic molecules which do not themselves comprise a first outer polar group, an apolar core group, and a second outer polar group, wherein each of the first and second outer polar groups is linked to the apolar core group. Thus, the further molecules may include amphiphilic molecules such as conventional lipids, for instance phospholipids, fatty acids, fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids and polyketides.

The amphipathic molecules in the layer or membrane which comprise a first outer hydrophilic group, a hydrophobic core group, and a second outer hydrophilic group, need not be all of the same type. Rather, mixtures of such amphipathic molecules may be present.

The term "linked" as disclosed with respect to the amphipathic molecules defined herein means bonded, either directly, or via one or more further groups. The one or more further groups may be selected from linker groups, further hydrophilic groups (i.e. hydrophilic groups other than the first and second outer hydrophilic groups), and further hydrophobic groups (i.e. hydrophobic groups other than the hydrophobic core group). Thus, in each of the amphipathic molecules, the first outer hydrophilic group is bonded to the hydrophobic core group either directly or via one or more further groups, and the second outer hydrophilic group is bonded to the hydrophobic core group, either directly or via one or more further groups.

It is important that the first and second outer hydrophilic groups are both linked independently to the hydrophobic core group in this way, because this ensures that the molecule contains at least three distinct regions in terms of hydrophobicity, i.e. an inner hydrophobic region and two outer hydrophilic regions. Thus, it is important that the molecule is not one in which only one of the first and second outer hydrophilic groups is linked to the

hydrophobic core group, e.g. a molecule of type AAB in which the first and second hydrophilic groups are bonded to each other and only one of those groups is linked to the hydrophobic core. Rather, each of the first and second outer hydrophilic groups must be linked to the hydrophobic core group independently, to provide at least three distinct regions in the molecule in terms of hydrophobicity.

Generally, for the same reason, the first and second outer hydrophilic groups are independently linked to different regions of the hydrophobic core group, so that the first and second outer hydrophilic groups are spaced apart from one another to some extent by the hydrophobic core group.

Usually, therefore, the first and second outer hydrophilic groups are independently linked to different atoms of the hydrophobic core group. In a preferred embodiment, the first and second outer hydrophilic groups are linked to opposite ends of the hydrophobic core group.

As mentioned above, the amphipathic molecule may further comprise at least one additional hydrophobic or hydrophilic group, i.e. in addition to the first outer hydrophilic group, the hydrophobic core group, and the second outer hydrophilic group.

Thus, for instance, each of said amphipathic molecules may further comprise at least one additional hydrophobic group which is bonded to the first outer hydrophilic group or the second outer hydrophilic group.

The fact that the amphipathic molecules may further comprise one or more additional hydrophobic groups does not necessarily mean that the amphipathic molecule cannot adopt a triblock type configuration, i.e. a configuration in which the molecule still has three distinct regions in terms of hydrophobicity. For instance, when the amphipathic molecule has an additional hydrophobic group which is bonded to the first or second outer hydrophilic group, that additional hydrophobic group may be capable of folding inwards, to align itself with the hydrophobic core group. The resulting conformation of the amphipathic molecule still has "triblock character" because the additional hydrophobic group can fold inwards to essentially become part of a core hydrophobic region together with the hydrophobic core group; essentially, therefore, such an amphipathic molecule still has an inner hydrophobic region and two outer hydrophilic regions, and is therefore very useful for forming a membrane between the first and second volumes of polar medium in accordance with the method of the invention.

In some embodiments, each of the amphipathic molecules further comprises: a first additional hydrophobic group which is bonded to the first outer hydrophilic group, and a

second additional hydrophobic group which is bonded to the second outer hydrophilic group. Such amphipathic molecules include pentablock molecules of type BABAB, wherein each group B, which may be the same or different, is a hydrophobic group and each group A, which may be the same or different, is hydrophilic. Typically, each additional hydrophobic group is capable of aligning itself with the hydrophobic core group. As mentioned above, this means that the amphipathic molecule can retain "triblock character" and essentially therefore still have an inner hydrophobic region and two outer hydrophilic regions, which is very useful for the purpose of forming a membrane between the first and second volumes of polar medium in accordance with the method of the invention.

Usually, some or all of the amphipathic molecules are copolymer molecules comprising at least three polymer segments, wherein the hydrophobic core group is an inner hydrophobic polymer segment, B, and the first and second outer hydrophilic groups are first and second outer hydrophilic polymer segments, A_1 and A_2.

However, amphipathic molecules other than copolymers are also envisaged, such as, for instance, bipolar or bola lipids. They may be naturally occurring or synthetic in nature. Thus, each of the amphipathic molecules may be a bipolar lipid, which comprises two hydrophilic head groups bonded to opposite ends of a hydrophobic tail group. Each hydrophilic head group may optionally be bonded to at least one further hydrophobic tail group. Any suitable such bipolar lipid may be employed. Particularly suitable bipolar lipids include bipolar phospholipids. Examples of bipolar and bola lipids are macrocyclic tetraethers with two polar heads linked by two hydrophobic C40 phytanyl chains as found in Sulfolobus acidocaldarius, an extreme thermophilic archaebacterium, bipolar lipids such as disclosed by Brard *et al* J. Org. Chem., 2007, 72 (22), pp 8267–8279 and bola lipids such as disclosed by Schubert *et al* J. Phys. Chem. B 2008, 1212, 10041-10044.

Bipolar lipids can be synthesised using synthetic routes that are well known to the skilled chemist, and are also commercially available. The structure and the synthesis of various bipolar lipids is described in the review article "Archaeabacteria bipolar lipid analogues: structure, synthesis and lyotropic properties" Thierry Benvegnu et al., Current Opinion in Colloid & Interface Science, Volume 8, Issue 6, April 2004, Pages 469–479.

Usually, however, each of the amphipathic molecules is a copolymer comprising at least three polymer segments, wherein the hydrophobic core group is an inner hydrophobic polymer segment, B, and the first and second outer hydrophilic groups are first and second outer hydrophilic polymer segments, A_1 and A_2.

The copolymer may have for example a linear or graft structure. The first and second outer hydrophilic polymer segments, A_1 and A_2, may for instance be pendant from the inner hydrophobic polymer segment, B. Usually, A_1 and A_2, are linked to opposite ends of the inner hydrophobic polymer segment, B. As mentioned above, the term linked in this context means bonded, either directly, or via one or more further groups.

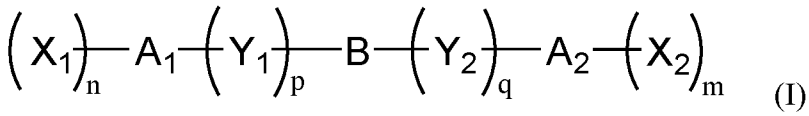
The copolymer may further comprise one or more additional polymer segments, i.e. one or more further polymer segments in addition to A_1, A_2 and B. The or each additional polymer segment may be the same or different. Typically, the or each additional polymer segment is an additional hydrophilic polymer segment or an additional hydrophobic polymer segment.

Thus, the first outer hydrophilic polymer segment A_1 may be bonded to one or more additional polymer segments. Likewise, the second outer hydrophilic polymer segment A_2 may be bonded to one or more additional polymer segments. In some embodiments, A_1 and A_2 are each bonded to one or more additional polymer segments.

Also, the inner hydrophobic polymer segment B may be bonded to the first outer hydrophilic polymer segment A_1 either directly or via one or more additional polymer segments. Likewise, the inner hydrophobic polymer segment B may be bonded to the second outer hydrophilic polymer segment A_2 directly, or via one or more additional polymer segments. In some embodiments, the inner hydrophobic polymer segment B is bonded to both A_1 and A_2, directly. However, other embodiments are envisaged in which the inner hydrophobic polymer segment B is bonded to both A_1 and A_2 via one or more additional polymer segments.

Each of these additional polymer segments may be independently selected from hydrophilic polymer segments and hydrophobic polymer segments.

Accordingly, the copolymer may be a block copolymer of formula (I)



(I)

wherein:

A_1 is said first outer hydrophilic polymer segment;

B is said inner hydrophobic polymer segment;

A_2 is said second outer hydrophilic polymer segment;

X_1, Y_1, Y_2 and X_2 are additional polymer segments; and

n, p, q and m are independently either 0 or 1.

The or each additional polymer segment, X_1, Y_1, Y_2 and X_2, may be the same or different. Each of these additional polymer segments may be a hydrophilic polymer segment or a hydrophobic polymer segment.

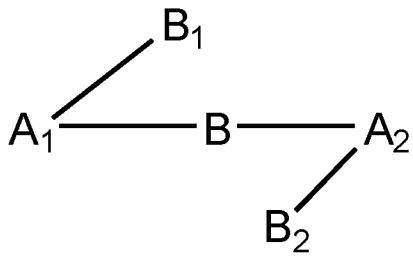
Usually, however, X_1 and X_2 are both additional hydrophilic polymer segments or X_1 and X_2 are both additional hydrophobic polymer segments.

Also, typically, Y_1 and Y_2 are both additional hydrophobic polymer segments or are both additional hydrophilic polymer segments.

In some embodiments, m and n in the block copolymer of formula (I) are both 1, and p and q are both 0, and the copolymer is therefore a pentablock copolymer. One preferred pentablock copolymer is a block copolymer of formula (I) in which m and n are both 1, and p and q are both 0, and X_1 and X_2 are both additional hydrophobic polymer segments.

Preferably, in this embodiment, the additional hydrophobic polymer segments X_1 and X_2 are capable of aligning themselves with the inner hydrophobic polymer segment B. For instance, X_1 and X_2 may be capable of folding inwards to align themselves with segment B. This means that the resulting conformation of the pentablock molecule still has "triblock character" because the hydrophobic polymer segments X_1 and X_2 essentially become part of a core hydrophobic region together with the hydrophobic core group. The copolymer still therefore has an inner hydrophobic region (comprising B and X_1 and X_2) and two outer hydrophilic regions, making it very useful for forming a membrane between the first and second volumes of polar medium in accordance with the method of the invention.

Thus, in some embodiments, the copolymer is a pentablock copolymer of formula (II):



(II)

wherein:

A_1 is said first outer hydrophilic polymer segment;

B is said inner hydrophobic polymer segment;

A_2 is said second outer hydrophilic polymer segment;

B_1 is a first additional hydrophobic polymer segment; and

B_2 is a second additional hydrophobic polymer segment.

B_1 and B_2, in this embodiment, are generally capable of folding inwards to align themselves with segment B, meaning that the pentablock molecule can adopt a conformation with "triblock character", with an inner hydrophobic region (comprising B, B_1 and B_2 aligned to each other) and two outer hydrophilic regions, A_1 and A_2, making the amphipathic molecule particularly useful for forming a membrane between the first and second volumes of a polar medium in accordance with the method of the invention.

Usually, however, the copolymer is a triblock copolymer having a middle polymer segment which is said inner hydrophobic polymer segment B, and two outer polymer segments which are said first and second outer hydrophilic polymer segments, A_1 and A_2.

Thus, typically, in formula (I), m, n, p and q are all 0, and the copolymer is a triblock copolymer of formula (III)

$$A_1-B-A_2 \qquad \text{(III)}$$

wherein

A_1 is said first outer hydrophilic polymer segment;

B is said inner hydrophobic polymer segment;

A_2 is said second outer hydrophilic polymer segment.

Usually, in this embodiment, A_1 and A_2, are bonded to opposite ends of the inner hydrophobic polymer segment, B.

The following substituent definitions apply with respect to the compounds defined hereinbelow:

A C_1-C_{18} alkyl group is an unsubstituted or substituted, straight or branched chain saturated hydrocarbon radical having from 1 to 18 carbon atoms. Typically it is C_1-C_{10} alkyl, for example methyl, ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, nonyl or decyl, or C_1-C_6 alkyl, for example methyl, ethyl, propyl, butyl, pentyl or hexyl, or C_1-C_4 alkyl, for example methyl, ethyl, i-propyl, n-propyl, t-butyl, s-butyl or n-butyl. The alkyl group may however be a C_3-C_{18} alkyl, or for instance a C_4-C_{12} alkyl group. When an alkyl group is substituted it typically bears one or more substituents selected from unsubstituted C_1-C_{10} alkyl, substituted or unsubstituted aryl (for instance phenyl), cyano, amino, C_1-C_{10} alkylamino, di(C_1-C_{10})alkylamino, arylamino, diarylamino, arylalkylamino, amido, acylamido, oxo, halo, ester, acyl, acyloxy, C_1-C_{10} alkoxy, aryloxy, haloalkyl, C_1-C_{10} alkylthio, sulfhydryl, arylthio,

sulfonyl, phosphate ester. Particularly if the alkyl group is within a hydrophilic group or within a hydrophilic polymer segment, it may bear one or more substituents selected from hydroxy, carboxy, sulfonic acid, phosphoric acid, and phosphonic acid. Examples of substituted alkyl groups include haloalkyl, hydroxyalkyl, aminoalkyl, alkoxyalkyl and alkaryl groups. The term alkaryl, as used herein, pertains to a C_1-C_{18} alkyl group in which at least one hydrogen atom has been replaced with an aryl group. Examples of such groups include, but are not limited to, benzyl (phenylmethyl, $PhCH_2$-), benzhydryl (Ph_2CH-), trityl (triphenylmethyl, Ph_3C-), phenethyl (phenylethyl, Ph-CH_2CH_2-), styryl (Ph-CH=CH-), cinnamyl (Ph-CH=CH-CH_2-). Typically a substituted C_1-C_{18} alkyl group carries 1, 2 or 3 substituents, for instance 1 or 2, or more typically 1 substituent. Usually, however, the alkyl groups herein are unsubstituted, unless otherwise specified.

A vinyl C_1-C_{18} alkanoate is therefore a compound of formula R-C(O)O-CH=CH_2, wherein R is a C_1-C_{18} alkyl group as defined above.

Unless otherwise specified an "alkyl" group specified herein may be taken to be a C_1-C_{18} alkyl group as defined above, or for instance a C_1-C_{10} alkyl group as defined above, or a C_1-C_4 alkyl group as defined above.

A C_1-C_{10} perfluoroalkyl group is a straight or branched chain saturated perfluorinated hydrocarbon radical having from 1 to 10 carbon atoms. A C_2-C_{10} perfluoroalkyl group is a straight or branched chain saturated perfluorinated hydrocarbon radical having from 2 to 10 carbon atoms. "Perfluorinated" in this context means completely fluorinated such that there are no carbon-bonded hydrogen atoms replaceable with fluorine. Examples of C_2-C_{12} perfluoro alkyl groups are perfluoroethyl (C_2) perfluoropropyl (C_3) (including perfluoro-*n*-propyl and perfluoro-*iso*-propyl), perfluorobutyl (C_4) (including perfluoro-*n*-butyl, perfluoro-*sec*-butyl and perfluoro-*tert*-butyl), perfluoropentyl (C_5), perfluorohexyl (C_6), perfluoroheptyl (C_7), perfluorooctyl (C_8), perfluorononyl (C_9), and perfluorodecyl (C_{10}), including straight chained and branched isomers thereof. C_1-C_{10} perfluoroalkyl also of course includes -CF_3.

"Partially fluorinated" means that one or more carbon-bonded hydrogen atoms are present which are replaceable with fluorine. Thus, a partially fluorinated C_1-C_{10} alkyl group is a C_1-C_{10} alkyl group which is substituted with one or more fluorine atoms but which is not perfluorinated. Likewise, a partially fluorinated C_2-C_{10} alkyl group is a C_2-C_{10} alkyl group which is substituted with one or more fluorine atoms but which is not perfluorinated. Thus, partially fluorinated C_1-C_{10} alkyl groups and C_2-C_{10} alkyl groups have at least one carbon-bonded hydrogen atom which is replaceable with fluorine.

A C_3-C_{10} cycloalkyl group is an unsubstituted or substituted alkyl group which is also a cyclyl group; that is, a monovalent moiety obtained by removing a hydrogen atom from an alicyclic ring atom of a carbocyclic ring of a carbocyclic compound, which moiety has from 3 to 10 carbon atoms (unless otherwise specified), including from 3 to 10 ring atoms. Examples of groups of C_3-C_{10} cycloalkyl groups include C_3-C_7 cycloalkyl. When a C_3-C_{10} cycloalkyl group is substituted it typically bears one or more substituents selected from those specified above for alkyl groups. Typically a substituted C_{3-10} cycloalkyl group carries 1, 2 or 3 substituents, for instance 1 or 2, or more typically 1 substituent. Usually, however, the cycloalkyl groups herein are unsubstituted unless otherwise specified. Examples of C_{3-10} cycloalkyl groups include, but are not limited to, those derived from saturated monocyclic hydrocarbon compounds, which C_{3-10} cycloalkyl groups are unsubstituted or substituted as defined above: cyclopropane (C_3), cyclobutane (C_4), cyclopentane (C_5), cyclohexane (C_6), cycloheptane (C_7), methylcyclopropane (C_4), dimethylcyclopropane (C_5), methylcyclobutane (C_5), dimethylcyclobutane (C_6), methylcyclopentane (C_6), dimethylcyclopentane (C_7), methylcyclohexane (C_7), dimethylcyclohexane (C_8), menthane (C_{10}).

An aryl group is a substituted or unsubstituted, monocyclic or bicyclic aromatic group which typically contains from 6 to 14 carbon atoms, preferably from 6 to 10 carbon atoms in the ring portion. Examples include phenyl, naphthyl, indenyl and indanyl groups. An aryl group may be unsubstituted or substituted, for instance, as specified above for alkyl. Typically it carries 0, 1, 2 or 3 substituents.

A C_2-C_{18} alkene is a straight or branched chain alkene having from 2 to 20 carbon atoms. A halo group is chlorine, fluorine, bromine or iodine (a chloro group, a fluoro group, a bromo group or an iodo group). It is typically chlorine, fluorine or bromine. A C_2-C_{18} haloalkene is therefore a straight or branched chain alkene having from 2 to 20 carbon atoms which is substituted with one or more halo groups. Typically it carries 0, 1, 2, 3 or 4 halo substituents.

As used herein the term amino represents a group of formula -NH_2. The term C_1-C_6 alkylamino represents a group of formula -NHR′ wherein R′ is a C_1-C_6 alkyl group, as defined previously. The term di(C_1-C_6 alkylamino) represents a group of formula -NR′R′′ wherein R′ and R′′ are the same or different and represent C_1-C_6 alkyl groups as defined previously.

The inner hydrophobic polymer segment B of the amphipathic copolymer molecules defined above typically comprises a polymer of one or more monomers selected from: C_1-C_{18} alkyl and C_3-C_{18} cycloalkyl acrylates and methacrylates, C_3-C_{18} alkylacrylamides and

methacrylamides, acrylonitrile, methacrylonitrile, vinyl C_1-C_{18} alkanoates, C_2-C_{18} alkenes, C_2-C_{18} haloalkenes, styrene, (C_{1-6} alkyl)styrene, C_4-C_{12} alkyl vinyl ethers, C_2-C_{10} perfluoro-alkyl acrylates and methacrylates and correspondingly partially fluorinated acrylates and methacrylates, C_3-C_{12} perfluoroalkylethylthiocarbonylaminoethyl acrylates and methacrylates, acryloxy- and methacryloxyalkylsiloxanes, di(C_1-C_6 alkyl)halosilane, N-vinylcarbazole, C_1-C_{12} alkyl esters of maleic acid, fumaric acid, itaconic acid, mesaconic acid, vinyl acetate, vinyl propionate, vinyl butyrate, vinyl valerate, chloroprene, vinyl chloride, vinylidene chloride, vinyltoluene, vinyl ethyl ether, perfluorohexyl ethylthiocarbonylaminoethyl methacrylate, isobomyl methacrylate, trifluoroethyl methacrylate, hexa-fluoroisopropyl methacrylate, hexafluorobutyl methacrylate, tristrimethylsilyloxysilylpropyl methacrylate (TRIS), and 3-methacryloxypropylpentamethyldisiloxane. Thus, the inner hydrophobic polymer segment B may comprise a polymer of any one of the monomers listed above, or it may comprise a copolymer of any two or more of the monomers listed above.

The inner hydrophobic polymer segment B may for instance comprise a polymer of one or more C_2-C_{18} alkene monomers, for instance a polymer of one or more C_2-C_4 alkene monomers.

Alternatively, the hydrophobic polymer segment B could for instance comprise a polymer of one or more di(C_1-C_6 alkyl)halosilane monomers, for instance a polymer of dimethylchlorosilane.

When one or more additional hydrophobic polymer segments are present in the copolymer, for instance when any of X_1, Y_1, Y_2 and X_2 is present in formula (I) above and is an additional hydrophobic polymer segment, or for instance when the copolymer is a pentablock copolymer of formula (II) above which comprises the additional hydrophobic polymer segments B_1 and B_2, the or each additional hydrophobic polymer segment, which may be the same or different, typically comprises a polymer of one or more monomers selected from: C_1-C_{18} alkyl and C_3-C_{18} cycloalkyl acrylates and methacrylates, C_3-C_{18} alkylacrylamides and methacrylamides, acrylonitrile, methacrylonitrile, vinyl C_1-C_{18} alkanoates, C_2-C_{18} alkenes, C_2-C_{18} haloalkenes, styrene, (C_{1-6} alkyl)styrene, C_4-C_{12} alkyl vinyl ethers, C_2-C_{10} perfluoro-alkyl acrylates and methacrylates and correspondingly partially fluorinated acrylates and methacrylates, C_3-C_{12} perfluoroalkylethylthiocarbonylaminoethyl acrylates and methacrylates, acryloxy- and methacryloxyalkylsiloxanes, N-vinylcarbazole, C_1-C_{12} alkyl esters of maleic acid, fumaric acid, itaconic acid, mesaconic acid, vinyl acetate, vinyl propionate, vinyl butyrate, vinyl valerate, chloroprene, vinyl chloride, vinylidene

chloride, vinyltoluene, vinyl ethyl ether, perfluorohexyl ethylthiocarbonylaminoethyl methacrylate, isobomyl methacrylate, trifluoroethyl methacrylate, hexa-fluoroisopropyl methacrylate, hexafluorobutyl methacrylate, tristrimethylsilyloxysilylpropyl methacrylate (TRIS), and 3-methacryloxypropylpentamethyldisiloxane. Thus, the or each additional hydrophobic polymer segment may comprise a polymer of any one of the monomers listed above, or may comprise a copolymer of any two or more of the monomers listed above.

The or each additional hydrophobic polymer segment may for instance comprise a polymer of one or more C_2-C_{18} alkene monomers, for instance a polymer of one or more C_2-C_4 alkene monomers. The or each additional hydrophobic polymer segment may additionally or alternatively comprise a polymer of one or more di(C_1-C_6 alkyl)halosilane monomers, for instance a polymer of dimethylchlorosilane.

Usually, the inner hydrophobic polymer segment B in the copolymer comprises a polymer selected from polysiloxane, polyalkene, perfluoropolyether, perfluoroalkyl polyether, polystyrene, polyoxypropylene, polyvinylacetate, polyoxybutylene, polyisoprene, polybutadiene, polyvinylchloride, polyalkylacrylate (PAA), polyalkylmethacrylate, polyacrylonitrile, polypropylene, PTHF, polymethacrylates, polyacrylates, polysulfones, polyvinylethers, poly(propylene oxide) and copolymers thereof.

Particularly preferred options for the inner hydrophobic polymer segment B include polysiloxane and polyalkene.

Suitable polysiloxanes include polydimethylsiloxane and polydiphenylsiloxane. The inner hydrophobic polymer segment B may for instance comprise a polysiloxane block having terminal alkylene groups. Thus, the inner hydrophobic polymer segment B may comprise a polydimethylsiloxane block having terminal alkylene groups, or for instance a polydiphenylsiloxane block having terminal alkylene groups.

Alternatively, the inner hydrophobic polymer segment B may comprise a polyalkene. The polyalkene may for instance be polyethylene, polypropylene, or polybutene. Typically, the polyalkene is polyethylene.

Similarly, when one or more additional hydrophobic polymer segments are present in the copolymer, for instance when any of X_1, Y_1, Y_2 and X_2 is present in formula (I) above and is an additional hydrophobic polymer segment, or for instance when the copolymer is a pentablock copolymer of formula (II) above which comprises the additional hydrophobic polymer segments B_1 and B_2, the or each additional hydrophobic polymer segment, which may be the same or different, typically comprises a polymer selected from polysiloxane, polyalkene, perfluoropolyether, perfluoroalkyl polyether, polystyrene, polyoxypropylene,

polyvinylacetate, polyoxybutylene, polyisoprene, polybutadiene, polyvinylchloride, polyalkylacrylate (PAA), polyalkylmethacrylate, polyacrylonitrile, polypropylene, PTHF, polymethacrylates, polyacrylates, polysulfones, polyvinylethers, poly(propylene oxide) and copolymers thereof. Particularly preferred options for the one or more additional hydrophobic polymer segments include polysiloxane and polyalkene. Suitable polysiloxanes include polydimethylsiloxane and polydiphenylsiloxane. The polyalkene may for instance be polyethylene, polypropylene or polybutene. Typically, the polyalkene is polyethylene.

Typically, therefore, the inner hydrophobic polymer segment B and, when present, the or each additional hydrophobic polymer segment, comprise a polysiloxane or a polyalkene. Suitable polysiloxanes include polydimethylsiloxane and polydiphenylsiloxane. The polyalkene may for instance be polyethylene, polypropylene, or polybutene.

In some embodiments, however, the inner hydrophobic polymer segment B comprises an unsaturated polymer. The unsaturated polymer may for instance be selected from: a polymer of a conjugated aliphatic or alicyclic diene, which diene is unsubstituted or substituted by halogen or C_1-C_6 alkyl; a polymer of an alkyne or dialkyne, which alkyne or dialkyne is unsubstituted or substituted by C_1-C_6 alkyl or trimethylsilyl; a copolymer of a conjugated diene and a hydrophilic or hydrophobic vinylic monomer; and partially hydrated derivatives thereof. Particularly preferred unsaturated polymers that may be used include: cis-, trans-, iso- or syndiotactic poly-1,2-butadiene, poly-1,4-butadiene or polyisoprene, poly-pentenamer, polychloroprene or polypiperylen; butadiene- or isoprene-copolymers with hydrophilic or hydrophobic vinylic monomers selected from acrylonitrile, styrene, acrylic acid, or hydroxyethylmethacrylate; or poly-1-trimethylsilyl-propyne.

When present, the or each additional hydrophobic polymer segment, which may be the same or different, may comprise an unsaturated polymer. When the or each additional hydrophobic polymer segment comprises an unsaturated polymer, the unsaturated polymer may for instance be selected from any of those listed above for the inner hydrophobic polymer segment B.

The inner hydrophobic polymer segment B and, when present, the or each additional hydrophobic polymer segment, may include a single type of polymer or more than one type of polymer, such as two or more of those discussed above.

The mean molecular weight of the inner hydrophobic polymer segment B is typically from about 150 to about 50,000. In some embodiments, it is from about 800 to about 15,000, or for instance from about 1,000 to about 12,000. In some embodiments, it is from about 5,000 to about 12,000, for instance from about 4,000 to about 11,000.

Likewise, the mean molecular weight of the or each additional hydrophobic polymer segment, when present, is typically from about 150 to about 50,000. In some embodiments, it is from about 800 to about 15,000, or for instance from about 1,000 to about 12,000. In some embodiments, it is from about 5,000 to about 12,000, for instance from about 4,000 to about 11,000.

The first outer hydrophilic polymer segment, A_1, and the second outer hydrophilic polymer segment, A_2, of the amphipathic copolymer molecules defined above may be the same or different. Usually, A_1 and A_2, which are the same or different, comprise a polymer of a monomer which is independently selected from: hydroxyl-substituted C_1-C_6 alkyl acrylates and methacrylates, acrylamide, methacrylamide, (C_1-C_6 alkyl)acrylamides and methacrylamides, N,N-dialkyl-acrylamides, ethoxylated acrylates and methacrylates, polyethyleneglycol-mono methacrylates and polyethyleneglycolmonomethylether methacrylates, hydroxyl-substituted (C_1-C_6 alkyl)acrylamides and methacrylamides, hydroxyl-substituted C_1-C_6 alkyl vinyl ethers, sodium vinylsulfonate, sodium styrenesulfonate, 2-acrylamido-2-methylpropanesulfonic acid, N-vinylpyrrole, N-vinyl-2-pyrrolidone, 2-vinyloxazoline, 2-vinyl-4,4'-dialkyloxazolin-5-one, 2- and 4-vinylpyridine, vinylically unsaturated carboxylic acids having a total of 3 to 5 carbon atoms, amino(C_1-C_6 alkyl)-, mono(C_1-C_6 alkylamino)(C_1-C_6 alkyl)- and di(C_1-C_6 alkylamino)(C_1-C_6 alkyl)-acrylates and methacrylates, allyl alcohol, 3-trimethylammonium 2-hydroxypropylmethacrylate chloride, dimethylaminoethyl methacrylate (DMAEMA), dimethylaminoethylmethacrylamide, glycerol methacrylate, N-(1,1-dimethyl-3-oxobutyl)acrylamide, cyclic imino ethers, vinyl ethers, cyclic ethers including epoxides, cyclic unsaturated ethers, N-substituted aziridines, [beta]-lactones and [beta]-lactames, ketene acetals, vinyl acetals and phosphoranes. Thus, each of the first and second outer hydrophilic polymer segments, A_1 and A_2, may comprise a polymer of any one of the monomers listed above, or a copolymer of any two or more of the monomers listed above.

The first outer hydrophilic polymer segment, A_1, and the second outer hydrophilic polymer segment, A_2, which are the same or different, may for instance comprise a polymer of a monomer which is independently selected from: a cyclic imino ether selected from 2-methyloxazoline, 2-oxazoline, and 2-oxazoline having an alkenyl group in the 2 position, and a vinyl ether selected from methyl vinyl ether, ethyl vinyl ether and methoxy ethyl vinyl ether. More typically, A_1 and A_2 comprise a polymer of a monomer selected from: 2-methyloxazoline, 2-oxazoline, and 2-oxazoline having an alkenyl group in the 2 position. For instance, one or both of A_1 and A_2 may comprise poly(2-methyloxazoline) (PMOXA).

Likewise, when one or more additional hydrophilic polymer segments are present in the copolymer, for instance when any of X_1, Y_1, Y_2 and X_2 is present in formula (I) above and is an additional hydrophilic polymer segment, the or each additional hydrophilic polymer segment, which may be the same or different, typically comprises a polymer of one or more monomers selected from: hydroxyl-substituted C_1-C_6 alkyl acrylates and methacrylates, acrylamide, methacrylamide, (C_1-C_6 alkyl)acrylamides and methacrylamides, N,N-dialkyl-acrylamides, ethoxylated acrylates and methacrylates, polyethyleneglycol-mono methacrylates and polyethyleneglycolmonomethylether methacrylates, hydroxyl-substituted (C_1-C_6 alkyl)acrylamides and methacrylamides, hydroxyl-substituted C_1-C_6 alkyl vinyl ethers, sodium vinylsulfonate, sodium styrenesulfonate, 2-acrylamido-2-methylpropanesulfonic acid, N-vinylpyrrole, N-vinyl-2-pyrrolidone, 2-vinyloxazoline, 2-vinyl-4,4'-dialkyloxazolin-5-one, 2- and 4-vinylpyridine, vinylically unsaturated carboxylic acids having a total of 3 to 5 carbon atoms, amino(C_1-C_6 alkyl)-, mono(C_1-C_6 alkylamino)(C_1-C_6 alkyl)- and di(C_1-C_6 alkylamino)(C_1-C_6 alkyl)- acrylates and methacrylates, allyl alcohol, 3-trimethylammonium 2-hydroxypropylmethacrylate chloride, dimethylaminoethyl methacrylate (DMAEMA), dimethylaminoethylmethacrylamide, glycerol methacrylate, N-(1,1-dimethyl-3-oxobutyl)acrylamide, cyclic imino ethers, vinyl ethers, cyclic ethers including epoxides, cyclic unsaturated ethers, N-substituted aziridines, [beta]-lactones and [beta]-lactames, ketene acetals, vinyl acetals and phosphoranes. Thus, the or each additional hydrophilic polymer segment, when present, may comprise a polymer of any one of the monomers listed above, or a copolymer of any two or more of the monomers listed above.

In some embodiments, the or each additional hydrophilic polymer segment, when present, comprises a polymer of a monomer selected from: a cyclic imino ether selected from 2-methyloxazoline, 2-oxazoline, and 2-oxazoline having an alkenyl group in the 2 position, and a vinyl ether selected from methyl vinyl ether, ethyl vinyl ether and methoxy ethyl vinyl ether. More typically, the or each additional hydrophilic polymer segment comprises a polymer of a monomer selected from: 2-methyloxazoline, 2-oxazoline, and 2-oxazoline having an alkenyl group in the 2 position. For instance, the or each additional hydrophilic polymer segment may comprise poly(2-methyloxazoline) (PMOXA).

Typically, the first outer hydrophilic polymer segment, A_1, and the second outer hydrophilic polymer segment, A_2, which are the same or different, comprise a polymer selected from: polyoxazoline, polyethylene glycol, polyethylene oxide, polyvinyl alcohol, polyvinylpyrrolidone, polyacrylamide, poly(meth)acrylic acid, polyethylene oxide-co-polypropyleneoxide block copolymers, poly (vinylether), poly(N,N-dimethylacrylamide),

polyacrylic acid, polyacyl alkylene imine, polyhydroxyalkylacrylates such as hydroxyethyl methacrylate (HEMA), hydroxyethyl acrylate, and hydroxypropyl acrylate, polyols, and copolymeric mixtures of two or more thereof, natural polymers such as polysaccharides and polypeptides, and copolymers thereof, and polyionic molecules such as polyallylammonium, polyethyleneimine, polyvinylbenzyltrimethylammonium, polyaniline, sulfonated polyaniline, polypyrrole, and polypyridinium, polythiophene-acetic acids, polystyrenesulfonic acids, zwitterionic molecules, and salts and copolymers thereof.

A particularly important choice of polymer for the hydrophilic polymer segments is poly(2-methyloxazoline), i.e. PMOXA. Thus, usually, the first outer hydrophilic polymer segment A_1 and the second outer hydrophilic polymer segment A_2 comprise poly(2-methyloxazoline).

Similarly, the or each additional hydrophilic polymer segment, when present, which may be the same or different when more than one additional hydrophilic polymer segment is present, comprises a polymer selected from: polyoxazoline, polyethylene glycol, polyethylene oxide, polyvinyl alcohol, polyvinylpyrrolidone, polyacrylamide, poly(meth)acrylic acid, polyethylene oxide-co-polypropyleneoxide block copolymers, poly (vinylether), poly(N,N-dimethylacrylamide), polyacrylic acid, polyacyl alkylene imine, polyhydroxyalkylacrylates such as hydroxyethyl methacrylate (HEMA), hydroxyethyl acrylate, and hydroxypropyl acrylate, polyols, and copolymeric mixtures of two or more thereof, natural polymers such as polysaccharides and polypeptides, and copolymers thereof, and polyionic molecules such as polyallylammonium, polyethyleneimine, polyvinylbenzyltrimethylammonium, polyaniline, sulfonated polyaniline, polypyrrole, and polypyridinium, polythiophene-acetic acids, polystyrenesulfonic acids, zwitterionic molecules, and salts and copolymers thereof. The additional hydrophilic polymer segment(s), when present, may for instance comprise poly(2-methyloxazoline), i.e. PMOXA.

The the first outer hydrophilic polymer segment A_1 and the second outer hydrophilic polymer segment A_2 and, when present, the or each additional hydrophilic polymer segment, may include a single type of polymer or more than one type of polymer, such as two or more of those discussed above.

The mean molecular weight of the first and second outer hydrophilic polymer segments A_1 and A_2 respectively, is typically from about 150 to about 50,000. In some embodiments, it is from about 500 to about 15,000, or for instance from about 1,000 to about 12,000. In some embodiments, it is from about 5,000 to about 12,000, for instance from about 4,000 to about 11,000.

Likewise, the mean molecular weight of the or each additional hydrophilic polymer segment, when present, is typically from about 150 to about 50,000. In some embodiments, it is from about 500 to about 15,000, or for instance from about 1,000 to about 12,000. In some embodiments, it is from about 5,000 to about 12,000, for instance from about 4,000 to about 11,000.

Thus, the molecular weight of each of the first outer hydrophilic polymer segment A_1, the second outer hydrophilic polymer segment A_2 and, when present, the or each additional hydrophilic polymer segment, is usually from 150 to 50,000. In some embodiments, it is from about 500 to about 15,000, or for instance from about 1,000 to about 12,000. In some embodiments, it is from about 5,000 to about 12,000, for instance from about 4,000 to about 11,000.

The amphipathic molecules may for instance comprise the triblock copolymer poly(2-methyloxazoline)-block-poly(dimethylsiloxane)-block-poly(2-methyloxazoline) (PMOXA-PDMS-PMOXA), or for instance the triblock copolymer poly(2-methyloxazoline)-block-poly(ethylene)-block-poly(2-methyloxazoline) (PMOXA-PE-PMOXA).

The amphipathic molecules may for instance comprise the triblock copolymer 6-33-6 (PMOXA-PDMS-PMOXA), 6-32-6 (PMOXA-PDMS-PMOXA), or 6-45PE-6 (PMOXA-PE-PMOXA).

Polymeric amphipathic molecules of the kind defined above, i.e. copolymer molecules comprising an inner hydrophobic polymer segment, and first and second outer hydrophilic polymer segments, may be synthesised using standard copolymer synthesis methods which are known in the art. Such methods are described in US 6,723,814 B2 and US 6,916,488 B1.

Any suitable polymerisation method can be used to prepare a hydrophobic or hydrophilic polymer segment as appropriate, including for instance photopolymerisation, redox polymerisation, anionic polymerisation, condensation reactions, addition reactions, and chain polymerisation reactions. Also, a wide variety of hydrophilic and hydrophobic polymers which can be used as segments in an amphipathic block copolymer are commercially available.

Hydrophilic and hydrophobic segments may be linked together by, for instance, polymerizing a suitable hydrophilic monomer in the presence of a suitably functionalized hydrophobic polymer segment, such that a block of units of the hydrophilic monomer grows from the site of functionalization of the hydrophobic segment. Alternatively a suitable hydrophobic monomer may be polymerised in the presence of a suitably functionalized

hydrophilic polymer segment, such that a block of units of the hydrophobic monomer grows from the site of functionalization of the hydrophilic segment.

Thus, for instance, a triblock copolymer may be prepared by polymerising one or more suitable hydrophilic monomers in the presence of a hydrophobic polymer segment which has been functionalised twice, such that two blocks of units of a hydrophilic monomer grow from the sites of functionalization of the hydrophobic segment.

The functionalized segment may be referred to as a macroinitiator. Suitable macroinitiators may bear one or more thermally or photochemically activatable cationic or anionic functional groups, or for instance one or more thermally or photochemically activatable radical initiator groups. Anionic polymerization, polycondensation, and polyaddition can also be used. Specific examples of preferred photochemically activatable cationic initiator groups are triflate (-O-SO_2-CF_3), -I (iodide), -O-mesyl, -O-tosyl, and -Cl^+AgSbF_6. The initiator group is usually linked to the starting segment in a way that provides a covalent bond between the terminal group of the starting segment and the first monomer forming the growing segment that is attached to the starting segment during the graft copolymerization for preparing the amphiphilic copolymer. Grafting means that polymer chains are grown from a monomer either in terminal or in pendant position onto another preformed polymer.

The initiator group may be introduced into a preformed polymer segment in any suitable way, for example through linkage of cationic or thermal initiator groups to functional groups present on the starting monomer. Triflate groups, for instance, can be introduced by reaction of terminal or pendent functional hydroxyl groups with activated triflic acid derivatives such as $(CF_3SO)_2O$.

It is also possible to change the monomer during graft copolymerization such that, for example, first hydrophilic segments A_1 and A_2 are grown on a preformed hydrophobic segment B and then further hydrophobic segments B_1 and B_2 are attached to the termini of the earlier prepared segments A_1 and A_2. Such a process could be used to a pentablock copolymer of formula (II) as defined herein.

The polymerizations can of course be carried out in the presence or absence of a solvent, and under appropriate conditions for the polymerisation reaction to take place, as are known to the skilled person. Suitable solvents are all solvents which dissolve the monomers used, for example, water, alcohols such as lower alkanols like ethanol or methanol, carboxamides such as dimethylformamide, dipolar aprotic solvents such as dimethyl sulfoxide or methyl ethyl ketone, ketones such as acetone or cyclohexanone, hydrocarbons

such as toluene, ethers such as THF, dimethoxyethane or dioxane, halogenated hydrocarbons such as trichloroethane, and mixtures of suitable solvents such as mixtures of water and an alcohol, for example, a water/ethanol or water/methanol mixture.

Complete copolymers comprising an inner hydrophobic polymer segment, and first and second outer hydrophilic polymer segments are also commercially available, from companies such as Polymer Source™, in Montreal, Canada, and for instance High Force Research Limited, Durham, UK.

The polar medium employed in the first and second volumes of polar medium (and in any further volumes of polar medium that are present) may be freely chosen for purpose. It is typically a liquid or a gel. The polar medium employed in the first and second volumes may be the same or different.

In the case that the polar medium is an aqueous medium. Any suitable aqueous medium may be employed. The aqueous medium may comprise one or more solutes. The aqueous medium may comprise a buffer in order to regulate the pH of the polar medium as appropriate.

The polar medium may further comprise a redox couple, or a member of a redox couple which may be partially oxidised or reduced to provide the redox couple. The redox couple may choen from those known in the art such as Fe^{2+}/Fe^{3+}, ferrocene/ferrocenium or Ru^{2+}/Ru^{3+}. Examples of such are ferro/ferricyanide, ruthenium hexamine and ferrocene carboxlic acid.

The apolar medium is typically an oil. The oil may be a single compound, or the oil may comprise a mixture of two or more compounds.

The oil may for instance comprise silicone oil. Suitable silicone oils include, for instance, poly(phenyl methyl siloxane) and poly(dimethylsiloxane) (PDMS). The silicone oil may comprise a hydroxy-terminated silicone oil, for instance hydroxy terminated PDMS.

The oil may comprise a single silicone oil, for instance poly(phenyl methyl siloxane) or poly(dimethylsiloxane). Alternatively, the oil may comprise a mixture of two or more different silicone oils, for instance a mixture of poly(phenyl methyl siloxane) and poly(dimethylsiloxane).

Additionally or alternatively, the oil may comprise a hydrocarbon, for instance hexadecane. When the oil comprises a hydrocarbon it may comprise a single hydrocarbon compound, or a mixture of two or more hydrocarbons. In some embodiments, the apolar medium is an oil which is a mixture comprising: (a) one or more hydrocarbons, and (b) one or more silicone oils.

Any suitable hydrocarbon may be employed as the oil. The hydrocarbon employed must of course be a liquid at the temperature of operation, i.e. at the temperature at which the method is perfomed. Typically, this is room temperature, and therefore the hydrocarbon employed will usually be one which is a liquid at room temperature.

When the oil comprises a hydrocarbon, the hydrocarbon may be branched or unbranched, for example a hydrocarbon having from 5 to 30 carbon atoms, or from 5 to 20 carbon atoms (although hydrocarbons of lower molecular weight would require control of evaporation). Preferably, the hydrocarbon is a liquid at the operating temperature of the droplet used in the invention. Suitable examples include alkanes or alkenes, such as hexadecane, decane, pentane or squalene. Usually, the oil comprises a hydrocarbon.

Typically the hydrocarbon is an unsubstituted C_{10}-C_{20} alkane, for instance hexadecane.

In some embodiments the hydrocarbon is a longer-chain hydrocarbon, such as unsubstituted C_{16}-C_{30} alkane, such as squalene.

Other types of oil are also possible. For example, the oil may be a fluorocarbon or a bromo-substituted C_{10}-C_{30} alkane (for instance a bromo-substituted C_{10}-C_{20} alkane, e.g. bromododecane). Typically, however, the oil comprises silicone oil or a hydrocarbon.

Silicone oil can be advantageous on account of its density being close to that of water, which ensures that a volume of polar medium which is an aqueous volume, is approximately neutrally buoyant in water. The silicone oil may for instance be poly(phenyl methyl siloxane), which has a density of about 1 $g.cm^{-3}$.

When a hydrocarbon is emplyed as the apolar medium the hydrocarbon typically has from 5 to 20 carbon atoms (a C_5-C_{20} hydrocarbon), more typically from 10 to 20 carbon atoms (a C_{10}-C_{20} hydrocarbon). Typically, it is an alkane or an alkene. Thus, the hydrocarbon may be a C_5-C_{20} alkane, or a C_{10}-C_{20} alkane. In another embodiment, the hydrocarbon may be a C_5-C_{20} alkene, or a C_{10}-C_{20} alkene. The hydrocarbon is typically unsubstituted. Thus, in a preferred embodiment, the hydrocarbon is an unsubstituted C_5-C_{20} alkane, preferably an unsubstituted C_{10}-C_{20} alkane. The hydrocarbon may for instance be squalene, hexadecane or decane. In one embodiment it is hexadecane. However, in some embodiments the hydrocarbon may be substituted with a halogen atom, for instance bromine.

The apolar medium may comprise a mixture of silicone oil and a hydrocarbon. The silicone oil and hydrocarbon in the mixture may be as further defined above. Typically, the hydrocarbon is an unsubstituted C_{10}-C_{20} alkane, preferably hexadecane. The silicone oil may for instance be poly(phenyl methyl siloxane) or PDMS.

In certain preferred embodiments of the method of the invention, the apolar medium comprises hexadecane, poly(phenyl methyl siloxane) or PDMS, the amphipathic molecules comprise poly(2-methyloxazoline)-block-poly(dimethylsiloxane)-block-poly(2-methyloxazoline) (PMOXA-PDMS-PMOXA), or poly(2-methyloxazoline)-block-poly(ethylene)-block-poly(2-methyloxazoline) (PMOXA-PE-PMOXA), and the polar medium comprises an aqueous buffer solution.

A membrane protein or a transmembrane pore may be provided in one or more of the volumes of polar medium, for insertion into the membrane or membranes that are formed between the volumes of polar medium by the method of the invention. The present method does not limit the choice of membrane protein. Thus, the membrane protein may be of any type. The use of integral membrane proteins has been demonstrated, but it is equally expected that peripheral membrane proteins could be used. The present method applies to any membrane proteins including the two major classes that are β-barrels or α-helical bundles. An important application is a membrane protein which is a pore or a channel. Besides a protein pore or channel, further possible membrane proteins include, but not exclusively, a receptor, a transporter or a protein which effects cell recognition or a cell-to-cell interaction.

Thus, typically, in the method of the invention for forming a membrane, at least one of the volumes of polar medium contains a membrane protein, which membrane protein is capable of insertion into the membrane or membranes comprising the amphipathic molecules. Suitable membrane proteins include, but are not limited to, pumps, channels (for instance ion channels) and/or pores, receptor proteins, transporter proteins, and/or proteins which effect cell recognition or a cell-to-cell interaction. Usually, the membrane protein is a pump, channel and/or pore.

Usually the membrane protein is a transmembrane pore, for instance MspA-(B2C), which is used in Example 2 hereinbelow, or for instance an α-hemolysin (αHL) pore. However, any suitable membrane protein can be used including the two major classes that is β-barrels or α-helical bundles.

Typically, the transmembrane protein pore is:

(a) selected from a hemolysin, leukocidin, *Mycobacterium smegmatis* porin A (MspA), outer membrane porin F (OmpF), outer membrane porin G (OmpG), outer membrane phospholipase A, *Neisseria* autotransporter lipoprotein (NalP) and WZA;

(b) formed of eight identical subunits as shown in SEQ ID NO: 2 or is a variant thereof in which one or more of the seven subunits has at least 50% homology to SEQ ID NO: 2 based on amino acid identity over the entire sequence and retains pore activity; or

(c) α-hemolysin formed of seven identical subunits as shown in SEQ ID NO: 4 or is a variant thereof in which one or more of the seven subunits has at least 50% homology to SEQ ID NO: 4 based on amino acid identity over the entire sequence and retains pore activity.

Usually, when a membrane protein is present in the polar medium, the concentration of the membrane protein in the polar medium is equal to or greater than 1 ng mL^{-1}, for instance, equal to or greater than 10 ng mL^{-1}. Typically, the concentration of membrane protein in the polar medium is from 10 ng mL^{-1} to 1000 ng mL^{-1}, or for instance from 200 ng mL^{-1} to 800 ng mL^{-1}.

Insertion of the pore into the membrane may be assisted by the presence of a surfactant. The surfactant advantageously has chemical moeities which are compatable with the copolymer. For example it has been shown that organosilicon based surfactants such as Silwet® can assist in the insertion of protein pores such as MspA into copolymers comprising siloxanes. The surfactant may be provided in the polar medium or the apolar medium.

The method of the invention for forming a membrane may further comprise taking a measurement on the volumes of polar medium to perform an experiment involving a process occurring at or through the membrane between the volumes. For instance, the method may further comprise bringing electrodes into electrical contact with the volumes of polar medium and taking an electrical measurement using the electrodes. Such measurements can be used to characterise a target analyte, as is explained further hereinbelow.

The various features of the system of the invention may be as further defined hereinbefore for the method of the invention.

In the system of the invention, the first volume of polar medium may be completely or partially within the apolar medium. In the case where the first volume of polar medium is partially within the apolar medium, a portion of it may not be in contact with the apolar medium. The membrane may thus be formed between a second volume comprising polar medium contacted with the exposed portion of the first volume.

Since the first volume comprising polar medium is within the apolar medium, either completely or partially, the system of the invention may further comprise a layer of the amphipathic molecules at an interface between the first volume of polar medium and the apolar medium.

The first volume of polar medium in the system of the invention is usually a droplet or bead. In some embodiments each of the first and second volumes in the system of the invention is a droplet or bead.

In some embodiments each of the first and second volumes of polar medium is within said apolar medium and the system further comprises: a layer of said amphipathic molecules at an interface between the first volume of polar medium and the apolar medium, and a layer of said amphipathic molecules at an interface between the second volume of polar medium and the apolar medium.

The system may additionally comprise one or more further volumes of polar medium, and one or more further membranes comprising said amphipathic molecules, wherein each further volume of polar medium is separated from another of the volumes of polar medium (which may be the first or second volume, or another further volume) by a said further membrane comprising said amphipathic molecules. The first volume, the second volume, and the one or more further volumes of polar medium may be droplets or beads.

The system may for instance comprise a further volume of polar medium adjacent to the first volume, and a further membrane comprising the amphipathic molecules between the first volume of polar medium and the further volume of polar medium.

Similarly, the system can comprise a further volume of polar medium adjacent to the second volume, and a further membrane comprising the amphipathic molecules between the second volume of polar medium and the further volume of polar medium.

One important setup is one in which the system comprises a plurality of first volumes of polar medium within the apolar medium and a plurality of respective membranes between the plurality of first volumes and the second volume. The or each first volume may be a droplet or bead. The second volume may comprise a sample comprising or suspected of comprising a target analyte of interest. The target analyte may be as further defined hereinbefore.

In another case, the system may comprise a plurality of first volumes of polar medium within the apolar medium, a plurality of second volumes of polar medium, and a plurality of membranes provided between the respective first and second volumes. The one or more second volumes may also be provided within the apolar medium.

The invention also provides a volume, as defined hereinbefore, comprising polar medium, which volume is disposed within a apolar medium, and which volume has a layer comprising the amphipathic molecules around a surface thereof, between the polar medium and the apolar medium. The volume may be usefully employed in the method of the invention as defined herein for forming a membrane. A process for producing the volume is also provided herein.

The various features of the volume of the invention, and the process of the invention for producing the volume, may be as further defined herein for the method of the invention for forming a membrane, or for the system of the invention. Thus, for instance, in the volume of the invention, or in the process of the invention for producing the volume of the invention, the amphipathic copolymer, the layer comprising the amphipathic molecules, the polar medium and the apolar medium, may all be as defined hereinbefore for the method or system of the invention. Usually, the volume of the polar medium is a droplet or bead of said polar medium.

Methods of characterising analytes

The invention provides a method of characterising a target analyte. The method comprises contacting the target analyte with a pore present in a membrane of the system of the invention such that the target analyte moves through the pore. One or more characteristics of the target analyte are then measured as the analyte moves with respect to the pore using standard methods known in the art. One or more characteristics of the target analyte are preferably measured as the analyte moves through the pore. Steps (a) and (b) are preferably carried out with a potential applied across the pore. As discussed in more detail below, the applied potential may results in the formation of a complex between the pore and a polynucleotide binding protein. The applied potential may be a voltage potential. Alternatively, the applied potential may be a chemical potential. An example of this is using a salt gradient across an amphiphilic layer. A salt gradient is disclosed in Holden *et al.*, J Am Chem Soc. 2007 Jul 11;129(27):8650-5.

The method of the invention is for characterising a target analyte. The method is for charaterising at least one analyte. The method may concern charaterising two or more analytes. The the method may comprise charaterising any number of analytes, such as 2, 5, 10, 15, 20, 30, 40, 50, 100 or more analytes.

The target analyte is preferably a metal ion, an inorganic salt, a polymer, an amino acid, a peptide, a polypeptide, a protein, a nucleotide, an oligonucleotide, a polynucleotide, a dye, a bleach, a pharmaceutical, a diagnostic agent, a recreational drug, an explosive or an environmental pollutant. The method may concern characterising two or more analytes of the same type, such as two or more proteins, two or more nucleotides or two or more pharmaceuticals. Alternatively, the method may concern characterising two or more analytes of different types, such as one or more proteins, one or more nucleotides and one or more pharmaceuticals.

The target analyte can be secreted from cells. Alternatively, the target analyte can be an analyte that is present inside cells such that the analyte must be extracted from the cells before the invention can be carried out.

The analyte is preferably an amino acid, a peptide, a polypeptides and/or a protein. The amino acid, peptide, polypeptide or protein can be naturally-occurring or non-naturally-occurring. The polypeptide or protein can include within them synthetic or modified amino acids. A number of different types of modification to amino acids are known in the art. Suitable amino acids and modifications thereof are above. For the purposes of the invention, it is to be understood that the target analyte can be modified by any method available in the art.

The protein can be an enzyme, an antibody, a hormone, a growth factor or a growth regulatory protein, such as a cytokine. The cytokine may be selected from interleukins, preferably IFN-1, IL-1, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 and IL-13, interferons, preferably IL-γ, and other cytokines such as TNF-α. The protein may be a bacterial protein, a fungal protein, a virus protein or a parasite-derived protein.

The target analyte is preferably a nucleotide, an oligonucleotide or a polynucleotide. A nucleotide typically contains a nucleobase, a sugar and at least one phosphate group. The nucleobase is typically heterocyclic. Nucleobases include, but are not limited to, purines and pyrimidines and more specifically adenine, guanine, thymine, uracil and cytosine. The sugar is typically a pentose sugar. Nucleotide sugars include, but are not limited to, ribose and deoxyribose. The nucleotide is typically a ribonucleotide or deoxyribonucleotide. The nucleotide typically contains a monophosphate, diphosphate or triphosphate. Phosphates may be attached on the 5' or 3' side of a nucleotide.

Nucleotides include, but are not limited to, adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), guanosine monophosphate (GMP), guanosine diphosphate (GDP), guanosine triphosphate (GTP), thymidine monophosphate (TMP), thymidine diphosphate (TDP), thymidine triphosphate (TTP), uridine monophosphate (UMP), uridine diphosphate (UDP), uridine triphosphate (UTP), cytidine monophosphate (CMP), cytidine diphosphate (CDP), cytidine triphosphate (CTP), 5-methylcytidine monophosphate, 5-methylcytidine diphosphate, 5-methylcytidine triphosphate, 5-hydroxymethylcytidine monophosphate, 5-hydroxymethylcytidine diphosphate, 5-hydroxymethylcytidine triphosphate, cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), deoxyadenosine monophosphate

(dAMP), deoxyadenosine diphosphate (dADP), deoxyadenosine triphosphate (dATP), deoxyguanosine monophosphate (dGMP), deoxyguanosine diphosphate (dGDP), deoxyguanosine triphosphate (dGTP), deoxythymidine monophosphate (dTMP), deoxythymidine diphosphate (dTDP), deoxythymidine triphosphate (dTTP), deoxyuridine monophosphate (dUMP), deoxyuridine diphosphate (dUDP), deoxyuridine triphosphate (dUTP), deoxycytidine monophosphate (dCMP), deoxycytidine diphosphate (dCDP) and deoxycytidine triphosphate (dCTP), 5-methyl-2'-deoxycytidine monophosphate, 5-methyl-2'-deoxycytidine diphosphate, 5-methyl-2'-deoxycytidine triphosphate, 5-hydroxymethyl-2'-deoxycytidine monophosphate, 5-hydroxymethyl-2'-deoxycytidine diphosphate and 5-hydroxymethyl-2'-deoxycytidine triphosphate. The nucleotides are preferably selected from AMP, TMP, GMP, UMP, dAMP, dTMP, dGMP or dCMP. The nucleotides may be abasic (i.e. lack a nucleobase). The nucleotides may contain additional modifications. In particular, suitable modified nucleotides include, but are not limited to, 2'amino pyrimidines (such as 2'-amino cytidine and 2'-amino uridine), 2'-hyrdroxyl purines (such as , 2'-fluoro pyrimidines (such as 2'-fluorocytidine and 2'fluoro uridine), hydroxyl pyrimidines (such as 5'-α-P-borano uridine), 2'-O-methyl nucelotides (such as 2'-O-methyl adenosine, 2'-O-methyl guanosine, 2'-O-methyl cytidine and 2'-O-methyl uridine), 4'-thio pyrimidines (such as 4'-thio uridine and 4'-thio cytidine) and nucleotides have modifiications of the nucleobase (such as 5-pentynyl-2'-deoxy uridine, 5-(3-aminopropyl)-uridine and 1,6-diaminohexyl-N-5-carbamoylmethyl uridine).

Oligonucleotides are short nucleotide polymers which typically have 50 or fewer nucleotides, such 40 or fewer, 30 or fewer, 20 or fewer, 10 or fewer or 5 or fewer nucleotides. The oligonucleotides may comprise any of the nucleotides discussed above, including the abasic and modified nucleotides.

The method of the invention is preferably for characterising a target polynucleotide. A polynucleotide, such as a nucleic acid, is a macromolecule comprising two or more nucleotides. The polynucleotide or nucleic acid may comprise any combination of any nucleotides. The nucleotides can be naturally occurring or artificial. One or more nucleotides in the target polynucleotide can be oxidized or methylated. One or more nucleotides in the target polynucleotide may be damaged. For instance, the polynucleotide may comprise a pyrimidine dimer. Such dimers are typically associated with damage by ultraviolet light and are the primary cause of skin melanomas. One or more nucleotides in the target polynucleotide may be modified, for instance with a label or a tag. Suitable labels are described above. The target polynucleotide may comprise one or more spacers.

Nucleotides are defined above. Nucleotides present in the polynucleotide typically include, but are not limited to, adenosine monophosphate (AMP), guanosine monophosphate (GMP), thymidine monophosphate (TMP), uridine monophosphate (UMP), cytidine monophosphate (CMP), cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), deoxyadenosine monophosphate (dAMP), deoxyguanosine monophosphate (dGMP), deoxythymidine monophosphate (dTMP), deoxyuridine monophosphate (dUMP) and deoxycytidine monophosphate (dCMP). The nucleotides are preferably selected from AMP, TMP, GMP, CMP, UMP, dAMP, dTMP, dGMP, dCMP and dUMP.

A nucleotide may be abasic (i.e. lack a nucleobase). A nucleotide may also lack a nucleobase and a sugar (i.e. is a C3 spacer).

The nucleotides in the polynucleotide may be attached to each other in any manner. The nucleotides are typically attached by their sugar and phosphate groups as in nucleic acids. The nucleotides may be connected via their nucleobases as in pyrimidine dimers.

The polynucleotide may be single stranded or double stranded. At least a portion of the polynucleotide is preferably double stranded. A single stranded polynucleotide may have one or more primers hybridised thereto and hence comprise one or more short regions of double stranded polynucleotide. The primers may be the same type of polynucleotide as the target polynucleotide or may be a different type of polynucleotide.

The polynucleotide can be a nucleic acid, such as deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). The target polynucleotide can comprise one strand of RNA hybridized to one strand of DNA. The polynucleotide may be any synthetic nucleic acid known in the art, such as peptide nucleic acid (PNA), glycerol nucleic acid (GNA), threose nucleic acid (TNA), locked nucleic acid (LNA) or other synthetic polymers with nucleotide side chains.

The whole or only part of the target polynucleotide may be characterised using this method. The target polynucleotide can be any length. For example, the polynucleotide can be at least 10, at least 50, at least 100, at least 150, at least 200, at least 250, at least 300, at least 400 or at least 500 nucleotide pairs in length. The polynucleotide can be 1000 or more nucleotide pairs, 5000 or more nucleotide pairs in length or 100000 or more nucleotide pairs in length.

The target analyte, such as a target polynucleotide, is present in any suitable sample. The invention is typically carried out on a sample that is known to contain or suspected to contain the target analyte, such as the target polynucleotide. Alternatively, the invention may

be carried out on a sample to confirm the identity of one or more target analytes, such as one or more target polynucleotides, whose presence in the sample is known or expected.

The sample may be a biological sample. The invention may be carried out *in vitro* on a sample obtained from or extracted from any organism or microorganism. The organism or microorganism is typically archaean, prokaryotic or eukaryotic and typically belongs to one the five kingdoms: plantae, animalia, fungi, monera and protista. The invention may be carried out *in vitro* on a sample obtained from or extracted from any virus. The sample is preferably a fluid sample. The sample typically comprises a body fluid of the patient. The sample may be urine, lymph, saliva, mucus or amniotic fluid but is preferably blood, plasma or serum. Typically, the sample is human in origin, but alternatively it may be from another mammal animal such as from commercially farmed animals such as horses, cattle, sheep or pigs or may alternatively be pets such as cats or dogs. Alternatively a sample of plant origin is typically obtained from a commercial crop, such as a cereal, legume, fruit or vegetable, for example wheat, barley, oats, canola, maize, soya, rice, bananas, apples, tomatoes, potatoes, grapes, tobacco, beans, lentils, sugar cane, cocoa, cotton.

The sample may be a non-biological sample. The non-biological sample is preferably a fluid sample. Examples of a non-biological sample include surgical fluids, water such as drinking water, sea water or river water, and reagents for laboratory tests.

The sample is typically processed prior to being assayed, for example by centrifugation or by passage through a membrane that filters out unwanted molecules or cells, such as red blood cells. The sample may be measured immediately upon being taken. The sample may also be typically stored prior to assay, preferably below -70°C.

The pore is present in the or a membrane of the system of the invention. Any of the embodiments discussed above with reference to the membrane of the system of the invention are applicable to the characterising method of the invention. The analyte, such as a target polynucleotide, may be coupled directly to the membrane. The analyte, such as a target polynucleotide, is preferably coupled to the membrane via a linker. Preferred linkers include, but are not limited to, polymers, such as polynucleotides, polyethylene glycols (PEGs) and polypeptides. If a polynucleotide is coupled directly to the membrane, then some data will be lost as the characterising run cannot continue to the end of the polynucleotide due to the distance between the membrane and the interior of the pore. If a linker is used, then the polynucleotide can be processed to completion. If a linker is used, the linker may be attached

to the polynucleotide at any position. The linker is preferably attached to the polynucleotide at the tail polymer.

The coupling may be stable or transient. For certain applications, the transient nature of the coupling is preferred. If a stable coupling molecule were attached directly to either the 5' or 3' end of a polynucleotide, then some data will be lost as the characterising run cannot continue to the end of the polynucleotide due to the distance between the bilayer and the interior of the pore. If the coupling is transient, then when the coupled end randomly becomes free of the bilayer, then the polynucleotide can be processed to completion. Chemical groups that form stable or transient links with the membrane are discussed in more detail below. The analyte, such as a target polynucleotide, may be transiently coupled to an amphiphilic layer, such as a lipid bilayer using cholesterol or a fatty acyl chain. Any fatty acyl chain having a length of from 6 to 30 carbon atoms, such as hexadecanoic acid, may be used.

Coupling of analytes, such as a target polynucleotide, to synthetic lipid bilayers has been carried out previously with various different tethering strategies. These are summarised in Table 1 below.

Table 1

Attachment group	**Type of coupling**	**Reference**
Thiol	Stable	Yoshina-Ishii, C. and S. G. Boxer (2003). "Arrays of mobile tethered vesicles on supported lipid bilayers." J Am Chem Soc 125(13): 3696-7.
Biotin	Stable	Nikolov, V., R. Lipowsky, et al. (2007). "Behavior of giant vesicles with anchored DNA molecules." Biophys J 92(12): 4356-68
Cholestrol	Transient	Pfeiffer, I. and F. Hook (2004). "Bivalent cholesterol-based coupling of oligonucletides to lipid membrane assemblies." J Am Chem Soc 126(33): 10224-5
Lipid	Stable	van Lengerich, B., R. J. Rawle, et al. "Covalent attachment of lipid vesicles to a fluid-supported bilayer allows observation of DNA-mediated vesicle interactions." Langmuir 26(11): 8666-72

Polynucleotides may be functionalized using a modified phosphoramidite in the synthesis reaction, which is easily compatible for the addition of reactive groups, such as thiol, cholesterol, lipid and biotin groups. These different attachment chemistries give a suite of attachment options for polynucleotides. Each different modification group tethers the polynucleotide in a slightly different way and coupling is not always permanent so giving different dwell times for the polynucleotide to the bilayer. The advantages of transient coupling are discussed above.

Coupling of polynucleotides can also be achieved by a number of other means provided that a reactive group can be added to the polynucleotide. The addition of reactive groups to either end of DNA has been reported previously. A thiol group can be added to the 5' of ssDNA using polynucleotide kinase and ATPγS (Grant, G. P. and P. Z. Qin (2007). "A facile method for attaching nitroxide spin labels at the 5' terminus of nucleic acids." Nucleic Acids Res 35(10): e77). A more diverse selection of chemical groups, such as biotin, thiols and fluorophores, can be added using terminal transferase to incorporate modified oligonucleotides to the 3' of ssDNA (Kumar, A., P. Tchen, et al. (1988). "Nonradioactive labeling of synthetic oligonucleotide probes with terminal deoxynucleotidyl transferase." Anal Biochem 169(2): 376-82).

Alternatively, the reactive group could be considered to be the addition of a short piece of DNA complementary to one already coupled to the bilayer, so that attachment can be achieved via hybridisation. Ligation of short pieces of ssDNA have been reported using T4 RNA ligase I (Troutt, A. B., M. G. McHeyzer-Williams, et al. (1992). "Ligation-anchored PCR: a simple amplification technique with single-sided specificity." Proc Natl Acad Sci U S A 89(20): 9823-5). Alternatively either ssDNA or dsDNA could be ligated to native dsDNA and then the two strands separated by thermal or chemical denaturation. To native dsDNA, it is possible to add either a piece of ssDNA to one or both of the ends of the duplex, or dsDNA to one or both ends. Then, when the duplex is melted, each single strand will have either a 5' or 3' modification if ssDNA was used for ligation or a modification at the 5' end, the 3' end or both if dsDNA was used for ligation. If the polynucleotide is a synthetic strand, the coupling chemistry can be incorporated during the chemical synthesis of the polynucleotide. For instance, the polynucleotide can be synthesized using a primer a reactive group attached to it.

A common technique for the amplification of sections of genomic DNA is using polymerase chain reaction (PCR). Here, using two synthetic oligonucleotide primers, a number of copies of the same section of DNA can be generated, where for each copy the 5'

of each strand in the duplex will be a synthetic polynucleotide. By using an antisense primer that has a reactive group, such as a cholesterol, thiol, biotin or lipid, each copy of the target DNA amplified will contain a reactive group for coupling.

A transmembrane pore is a structure that crosses the membrane to some degree. It permits hydrated ions driven by an applied potential to flow across or within the membrane. The transmembrane pore typically crosses the entire membrane so that hydrated ions may flow from one side of the membrane to the other side of the membrane. However, the transmembrane pore does not have to cross the membrane. It may be closed at one end. For instance, the pore may be a well in the membrane along which or into which hydrated ions may flow.

Any transmembrane pore may be used in the invention. The pore may be biological or artificial. Suitable pores include, but are not limited to, protein pores and polynucleotide pores.

The transmembrane pore is preferably a transmembrane protein pore. A transmembrane protein pore is a polypeptide or a collection of polypeptides that permits hydrated ions, such as analyte, to flow across or within the membrane. In the present invention, the transmembrane protein pore is preferably capable of forming a pore that permits hydrated ions driven by an applied potential to flow from one side of the membrane to the other. The transmembrane protein pore permits analytes, such as nucleotides, to flow from one side of the membrane to the other. The transmembrane protein pore allows a polynucleotide, such as DNA or RNA, to be moved through the pore.

The transmembrane protein pore may be a monomer or an oligomer. The pore is preferably made up of several repeating subunits, such as 6, 7, 8 or 9 subunits. The pore is preferably a hexameric, heptameric, octameric or nonameric pore.

The transmembrane protein pore typically comprises a barrel or channel through which the ions may flow. The subunits of the pore typically surround a central axis and contribute strands to a transmembrane β barrel or channel or a transmembrane α-helix bundle or channel.

The barrel or channel of the transmembrane protein pore typically comprises amino acids that facilitate interaction with analyte, such as nucleotides, polynucleotides or nucleic acids. These amino acids are preferably located near a constriction of the barrel or channel. The transmembrane protein pore typically comprises one or more positively charged amino acids, such as arginine, lysine or histidine, or aromatic amino acids, such as tyrosine or

tryptophan. These amino acids typically facilitate the interaction between the pore and nucleotides, polynucleotides or nucleic acids.

Transmembrane protein pores for use in accordance with the invention can be derived from β-barrel pores or α-helix bundle pores. β-barrel pores comprise a barrel or channel that is formed from β-strands. Suitable β-barrel pores include, but are not limited to, β-toxins, such as α-hemolysin, anthrax toxin and leukocidins, and outer membrane proteins/porins of bacteria, such as *Mycobacterium smegmatis* porin (Msp), for example MspA MspB, MspC or MspD, outer membrane porin F (OmpF), outer membrane porin G (OmpG), outer membrane phospholipase A and *Neisseria* autotransporter lipoprotein (NalP). α-helix bundle pores comprise a barrel or channel that is formed from α-helices. Suitable α-helix bundle pores include, but are not limited to, inner membrane proteins and α outer membrane proteins, such as WZA and ClyA toxin. The transmembrane pore may be derived from Msp or from α-hemolysin (α-HL).

The transmembrane protein pore is preferably derived from Msp, preferably from MspA. Such a pore will be oligomeric and typically comprises 7, 8, 9 or 10 monomers derived from Msp. The pore may be a homo-oligomeric pore derived from Msp comprising identical monomers. Alternatively, the pore may be a hetero-oligomeric pore derived from Msp comprising at least one monomer that differs from the others. Preferably the pore is derived from MspA or a homolog or paralog thereof.

A monomer derived from Msp typically comprises the sequence shown in SEQ ID NO: 2 or a variant thereof. SEQ ID NO: 2 is the MS-(B1)8 mutant of the MspA monomer. It includes the following mutations: D90N, D91N, D93N, D118R, D134R and E139K. A variant of SEQ ID NO: 2 is a polypeptide that has an amino acid sequence which varies from that of SEQ ID NO: 2 and which retains its ability to form a pore. The ability of a variant to form a pore can be assayed using any method known in the art. For instance, the variant may be inserted into an amphiphilic layer along with other appropriate subunits and its ability to oligomerise to form a pore may be determined. Methods are known in the art for inserting subunits into membranes, such as amphiphilic layers. For example, subunits may be suspended in a purified form in a solution containing a lipid bilayer such that it diffuses to the lipid bilayer and is inserted by binding to the lipid bilayer and assembling into a functional state. Alternatively, subunits may be directly inserted into the membrane using the "pick and place" method described in M.A. Holden, H. Bayley. J. Am. Chem. Soc. 2005, 127, 6502-

6503 and International Application No. PCT/GB2006/001057 (published as WO 2006/100484).

Over the entire length of the amino acid sequence of SEQ ID NO: 2, a variant will preferably be at least 50% homologous to that sequence based on amino acid identity. More preferably, the variant may be at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% and more preferably at least 95%, 97% or 99% homologous based on amino acid identity to the amino acid sequence of SEQ ID NO: 2 over the entire sequence. There may be at least 80%, for example at least 85%, 90% or 95%, amino acid identity over a stretch of 100 or more, for example 125, 150, 175 or 200 or more, contiguous amino acids ("hard homology").

Standard methods in the art may be used to determine homology. For example the UWGCG Package provides the BESTFIT program which can be used to calculate homology, for example used on its default settings (Devereux *et al* (1984) *Nucleic Acids Research* **12**, p387-395). The PILEUP and BLAST algorithms can be used to calculate homology or line up sequences (such as identifying equivalent residues or corresponding sequences (typically on their default settings)), for example as described in Altschul S. F. (1993) J Mol Evol 36:290-300; Altschul, S.F *et al* (1990) J Mol Biol 215:403-10. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

SEQ ID NO: 2 is the MS-(B1)8 mutant of the MspA monomer. The variant may comprise any of the mutations in the MspB, C or D monomers compared with MspA. The mature forms of MspB, C and D are shown in SEQ ID NOs: 5 to 7. In particular, the variant may comprise the following substitution present in MspB: A138P. The variant may comprise one or more of the following substitutions present in MspC: A96G, N102E and A138P. The variant may comprise one or more of the following mutations present in MspD: Deletion of G1, L2V, E5Q, L8V, D13G, W21A, D22E, K47T, I49H, I68V, D91G, A96Q, N102D, S103T, V104I, S136K and G141A. The variant may comprise combinations of one or more of the mutations and substitutions from Msp B, C and D. The variant preferably comprises the mutation L88N. A variant of SEQ ID NO: 2 has the mutation L88N in addition to all the mutations of MS-B1 and is called MS-(B2)8. The pore used in the invention is preferably MS-(B2)8. A variant of SEQ ID NO: 2 has the mutations G75S/G77S/L88N/Q126R in addition to all the mutations of MS-B1 and is called MS-B2C. The pore used in the invention is preferably MS-(B2)8 or MS-(B2C)8.

Amino acid substitutions may be made to the amino acid sequence of SEQ ID NO: 2 in addition to those discussed above, for example up to 1, 2, 3, 4, 5, 10, 20 or 30 substitutions. Conservative substitutions replace amino acids with other amino acids of similar chemical structure, similar chemical properties or similar side-chain volume. The amino acids introduced may have similar polarity, hydrophilicity, apolarity, basicity, acidity, neutrality or charge to the amino acids they replace. Alternatively, the conservative substitution may introduce another amino acid that is aromatic or aliphatic in the place of a pre-existing aromatic or aliphatic amino acid. Conservative amino acid changes are well-known in the art and may be selected in accordance with the properties of the 20 main amino acids as defined in Table 2 below. Where amino acids have similar polarity, this can also be determined by reference to the hydropathy scale for amino acid side chains in Table 2.

Table 2 – Chemical properties of amino acids

Ala	aliphatic, hydrophobic, neutral	Met	hydrophobic, neutral
Cys	polar, hydrophobic, neutral	Asn	polar, hydrophilic, neutral
Asp	polar, hydrophilic, charged (-)	Pro	hydrophobic, neutral
Glu	polar, hydrophilic, charged (-)	Gln	polar, hydrophilic, neutral
Phe	aromatic, hydrophobic, neutral	Arg	polar, hydrophilic, charged (+)
Gly	aliphatic, neutral	Ser	polar, hydrophilic, neutral
His	aromatic, polar, hydrophilic, charged (+)	Thr	polar, hydrophilic, neutral
Ile	aliphatic, hydrophobic, neutral	Val	aliphatic, hydrophobic, neutral
Lys	polar, hydrophilic, charged(+)	Trp	aromatic, hydrophobic, neutral
Leu	aliphatic, hydrophobic, neutral	Tyr	aromatic, polar, hydrophobic

Table 3 - Hydropathy scale

Side Chain	Hydropathy
Ile	4.5
Val	4.2
Leu	3.8
Phe	2.8

Cys	2.5
Met	1.9
Ala	1.8
Gly	-0.4
Thr	-0.7
Ser	-0.8
Trp	-0.9
Tyr	-1.3
Pro	-1.6
His	-3.2
Glu	-3.5
Gln	-3.5
Asp	-3.5
Asn	-3.5
Lys	-3.9
Arg	-4.5

One or more amino acid residues of the amino acid sequence of SEQ ID NO: 2 may additionally be deleted from the polypeptides described above. Up to 1, 2, 3, 4, 5, 10, 20 or 30 residues may be deleted, or more.

Variants may include fragments of SEQ ID NO: 2. Such fragments retain pore forming activity. Fragments may be at least 50, 100, 150 or 200 amino acids in length. Such fragments may be used to produce the pores. A fragment preferably comprises the pore forming domain of SEQ ID NO: 2. Fragments must include one of residues 88, 90, 91, 105, 118 and 134 of SEQ ID NO: 2. Typically, fragments include all of residues 88, 90, 91, 105, 118 and 134 of SEQ ID NO: 2.

One or more amino acids may be alternatively or additionally added to the polypeptides described above. An extension may be provided at the amino terminal or carboxy terminal of the amino acid sequence of SEQ ID NO: 2 or polypeptide variant or fragment thereof. The extension may be quite short, for example from 1 to 10 amino acids in length. Alternatively, the extension may be longer, for example up to 50 or 100 amino acids. A carrier protein may be fused to an amino acid sequence according to the invention. Other fusion proteins are discussed in more detail below.

As discussed above, a variant is a polypeptide that has an amino acid sequence which varies from that of SEQ ID NO: 2 and which retains its ability to form a pore. A variant typically contains the regions of SEQ ID NO: 2 that are responsible for pore formation. The pore forming ability of Msp, which contains a β-barrel, is provided by β-sheets in each subunit. A variant of SEQ ID NO: 2 typically comprises the regions in SEQ ID NO: 2 that form β-sheets. One or more modifications can be made to the regions of SEQ ID NO: 2 that form β-sheets as long as the resulting variant retains its ability to form a pore. A variant of SEQ ID NO: 2 preferably includes one or more modifications, such as substitutions, additions or deletions, within its α-helices and/or loop regions.

The monomers derived from Msp may be modified to assist their identification or purification, for example by the addition of histidine residues (a hist tag), aspartic acid residues (an asp tag), a streptavidin tag or a flag tag, or by the addition of a signal sequence to promote their secretion from a cell where the polypeptide does not naturally contain such a sequence. An alternative to introducing a genetic tag is to chemically react a tag onto a native or engineered position on the pore. An example of this would be to react a gel-shift reagent to a cysteine engineered on the outside of the pore. This has been demonstrated as a method for separating hemolysin hetero-oligomers (Chem Biol. 1997 Jul; 4(7):497-505).

The monomer derived from Msp may be labelled with a revealing label. The revealing label may be any suitable label which allows the pore to be detected. Suitable labels are described below.

The monomer derived from Msp may also be produced using D-amino acids. For instance, the monomer derived from Msp may comprise a mixture of L-amino acids and D-amino acids. This is conventional in the art for producing such proteins or peptides.

The monomer derived from Msp contains one or more specific modifications to facilitate nucleotide discrimination. The monomer derived from Msp may also contain other non-specific modifications as long as they do not interfere with pore formation. A number of non-specific side chain modifications are known in the art and may be made to the side chains of the monomer derived from Msp. Such modifications include, for example, reductive alkylation of amino acids by reaction with an aldehyde followed by reduction with $NaBH_4$, amidination with methylacetimidate or acylation with acetic anhydride.

The monomer derived from Msp can be produced using standard methods known in the art. The monomer derived from Msp may be made synthetically or by recombinant means. For example, the pore may be synthesized by *in vitro* translation and transcription (IVTT).

Suitable methods for producing pores are discussed in International Application Nos. PCT/GB09/001690 (published as WO 2010/004273), PCT/GB09/001679 (published as WO 2010/004265) or PCT/GB10/000133 (published as WO 2010/086603). Methods for inserting pores into membranes are discussed above.

The transmembrane protein pore is also preferably derived from α-hemolysin (α-HL). The wild type α -HL pore is formed of seven identical monomers or subunits (i.e. it is heptameric). The sequence of one monomer or subunit of α-hemolysin-NN is shown in SEQ ID NO: 4. The transmembrane protein pore preferably comprises seven monomers each comprising the sequence shown in SEQ ID NO: 4 or a variant thereof. Amino acids 1, 7 to 21, 31 to 34, 45 to 51, 63 to 66, 72, 92 to 97, 104 to 111, 124 to 136, 149 to 153, 160 to 164, 173 to 206, 210 to 213, 217, 218, 223 to 228, 236 to 242, 262 to 265, 272 to 274, 287 to 290 and 294 of SEQ ID NO: 4 form loop regions. Residues 113 and 147 of SEQ ID NO: 4 form part of a constriction of the barrel or channel of α-HL.

In such embodiments, a pore comprising seven proteins or monomers each comprising the sequence shown in SEQ ID NO: 4 or a variant thereof are preferably used in the method of the invention. The seven proteins may be the same (homo-heptamer) or different (hetero-heptamer).

A variant of SEQ ID NO: 4 is a protein that has an amino acid sequence which varies from that of SEQ ID NO: 4 and which retains its pore forming ability. The ability of a variant to form a pore can be assayed using any method known in the art. For instance, the variant may be inserted into an amphiphilic layer, such as a lipid bilayer, along with other appropriate subunits and its ability to oligomerise to form a pore may be determined. Methods are known in the art for inserting subunits into amphiphilic layers, such as lipid bilayers. Suitable methods are discussed above.

The variant may include modifications that facilitate covalent attachment to or interaction with the construct. The variant preferably comprises one or more reactive cysteine residues that facilitate attachment to the construct. For instance, the variant may include a cysteine at one or more of positions 8, 9, 17, 18, 19, 44, 45, 50, 51, 237, 239 and 287 and/or on the amino or carboxy terminus of SEQ ID NO: 4. Preferred variants comprise a substitution of the residue at position 8, 9, 17, 237, 239 and 287 of SEQ ID NO: 4 with cysteine (A8C, T9C, N17C, K237C, S239C or E287C). The variant is preferably any one of the variants described in International Application No. PCT/GB09/001690 (published as WO

2010/004273), PCT/GB09/001679 (published as WO 2010/004265) or PCT/GB10/000133 (published as WO 2010/086603).

The variant may also include modifications that facilitate any interaction with nucleotides.

The variant may be a naturally occurring variant which is expressed naturally by an organism, for instance by a *Staphylococcus* bacterium. Alternatively, the variant may be expressed *in vitro* or recombinantly by a bacterium such as *Escherichia coli*. Variants also include non-naturally occurring variants produced by recombinant technology. Over the entire length of the amino acid sequence of SEQ ID NO: 4, a variant will preferably be at least 50% homologous to that sequence based on amino acid identity. More preferably, the variant polypeptide may be at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% and more preferably at least 95%, 97% or 99% homologous based on amino acid identity to the amino acid sequence of SEQ ID NO: 4 over the entire sequence. There may be at least 80%, for example at least 85%, 90% or 95%, amino acid identity over a stretch of 200 or more, for example 230, 250, 270 or 280 or more, contiguous amino acids ("hard homology"). Homology can be determined as discussed above.

Amino acid substitutions may be made to the amino acid sequence of SEQ ID NO: 4 in addition to those discussed above, for example up to 1, 2, 3, 4, 5, 10, 20 or 30 substitutions. Conservative substitutions may be made as discussed above.

One or more amino acid residues of the amino acid sequence of SEQ ID NO: 4 may additionally be deleted from the polypeptides described above. Up to 1, 2, 3, 4, 5, 10, 20 or 30 residues may be deleted, or more.

Variants may be fragments of SEQ ID NO: 4. Such fragments retain pore-forming activity. Fragments may be at least 50, 100, 200 or 250 amino acids in length. A fragment preferably comprises the pore-forming domain of SEQ ID NO: 4. Fragments typically include residues 119, 121, 135. 113 and 139 of SEQ ID NO: 4.

One or more amino acids may be alternatively or additionally added to the polypeptides described above. An extension may be provided at the amino terminus or carboxy terminus of the amino acid sequence of SEQ ID NO: 4 or a variant or fragment thereof. The extension may be quite short, for example from 1 to 10 amino acids in length. Alternatively, the extension may be longer, for example up to 50 or 100 amino acids. A carrier protein may be fused to a pore or variant.

As discussed above, a variant of SEQ ID NO: 4 is a subunit that has an amino acid sequence which varies from that of SEQ ID NO: 4 and which retains its ability to form a pore. A variant typically contains the regions of SEQ ID NO: 4 that are responsible for pore formation. The pore forming ability of α-HL, which contains a β-barrel, is provided by β-strands in each subunit. A variant of SEQ ID NO: 4 typically comprises the regions in SEQ ID NO: 4 that form β-strands. The amino acids of SEQ ID NO: 4 that form β-strands are discussed above. One or more modifications can be made to the regions of SEQ ID NO: 4 that form β-strands as long as the resulting variant retains its ability to form a pore. Specific modifications that can be made to the β-strand regions of SEQ ID NO: 4 are discussed above.

A variant of SEQ ID NO: 4 preferably includes one or more modifications, such as substitutions, additions or deletions, within its α-helices and/or loop regions. Amino acids that form α-helices and loops are discussed above.

The variant may be modified to assist its identification or purification as discussed above.

Pores derived from α-HL can be made as discussed above with reference to pores derived from Msp.

In some embodiments, the transmembrane protein pore is chemically modified. The pore can be chemically modified in any way and at any site. The transmembrane protein pore is preferably chemically modified by attachment of a molecule to one or more cysteines (cysteine linkage), attachment of a molecule to one or more lysines, attachment of a molecule to one or more non-natural amino acids, enzyme modification of an epitope or modification of a terminus. Suitable methods for carrying out such modifications are well-known in the art. The transmembrane protein pore may be chemically modified by the attachment of any molecule. For instance, the pore may be chemically modified by attachment of a dye or a fluorophore.

Any number of the monomers in the pore may be chemically modified. One or more, such as 2, 3, 4, 5, 6, 7, 8, 9 or 10, of the monomers is preferably chemically modified as discussed above.

The reactivity of cysteine residues may be enhanced by modification of the adjacent residues. For instance, the basic groups of flanking arginine, histidine or lysine residues will change the pKa of the cysteines thiol group to that of the more reactive S^- group. The reactivity of cysteine residues may be protected by thiol protective groups such as dTNB.

These may be reacted with one or more cysteine residues of the pore before a linker is attached.

The molecule (with which the pore is chemically modified) may be attached directly to the pore or attached via a linker as disclosed in International Application Nos. PCT/GB09/001690 (published as WO 2010/004273), PCT/GB09/001679 (published as WO 2010/004265) or PCT/GB10/000133 (published as WO 2010/086603).

Any of the proteins described herein, such as the transmembrane protein pores, may be modified to assist their identification or purification, for example by the addition of histidine residues (a his tag), aspartic acid residues (an asp tag), a streptavidin tag, a flag tag, a SUMO tag, a GST tag or a MBP tag, or by the addition of a signal sequence to promote their secretion from a cell where the polypeptide does not naturally contain such a sequence. An alternative to introducing a genetic tag is to chemically react a tag onto a native or engineered position on the pore or construct. An example of this would be to react a gel-shift reagent to a cysteine engineered on the outside of the pore. This has been demonstrated as a method for separating hemolysin hetero-oligomers (Chem Biol. 1997 Jul;4(7):497-505).

The pore may be labelled with a revealing label. The revealing label may be any suitable label which allows the pore to be detected. Suitable labels include, but are not limited to, fluorescent molecules, radioisotopes, e.g. ^{125}I, ^{35}S, enzymes, antibodies, antigens, polynucleotides and ligands such as biotin.

Any of the proteins described herein, such as the transmembrane protein pores, may be made synthetically or by recombinant means. For example, the pore may be synthesized by *in vitro* translation and transcription (IVTT). The amino acid sequence of the pore may be modified to include non-naturally occurring amino acids or to increase the stability of the protein. When a protein is produced by synthetic means, such amino acids may be introduced during production. The pore may also be altered following either synthetic or recombinant production.

The pore may also be produced using D-amino acids. For instance, the pore or construct may comprise a mixture of L-amino acids and D-amino acids. This is conventional in the art for producing such proteins or peptides.

The pore may also contain other non-specific modifications as long as they do not interfere with pore formation or construct function. A number of non-specific side chain modifications are known in the art and may be made to the side chains of the protein(s). Such modifications include, for example, reductive alkylation of amino acids by reaction with

an aldehyde followed by reduction with $NaBH_4$, amidination with methylacetimidate or acylation with acetic anhydride.

Any of the proteins described herein, such as the transmembrane protein pores, can be produced using standard methods known in the art. Polynucleotide sequences encoding a pore or construct may be derived and replicated using standard methods in the art. Polynucleotide sequences encoding a pore or construct may be expressed in a bacterial host cell using standard techniques in the art. The pore may be produced in a cell by *in situ* expression of the polypeptide from a recombinant expression vector. The expression vector optionally carries an inducible promoter to control the expression of the polypeptide. These methods are described in Sambrook, J. and Russell, D. (2001). Molecular Cloning: A Laboratory Manual, 3rd Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

The pore may be produced in large scale following purification by any protein liquid chromatography system from protein producing organisms or after recombinant expression. Typical protein liquid chromatography systems include FPLC, AKTA systems, the Bio-Cad system, the Bio-Rad BioLogic system and the Gilson HPLC system.

The method is preferably for characterising a target polynucleotide and step (a) comprises contacting the target polynucleotide with the pore and a polynucleotide binding protein and the protein controls the movement of the target polynucleotide through the pore. The target polynucleotide may be contacted with the pore and the polynucleotide binding protein in any order. In is preferred that, when the target polynucleotide is contacted with the protein and the pore, the target polynucleotide firstly forms a complex with the protein. When the voltage is applied across the pore, the target polynucleotide/protein complex then forms a complex with the pore and controls the movement of the polynucleotide through the pore.

The polynucleotide binding protein may be any protein that is capable of binding to the polynucleotide and controlling its movement through the pore. It is straightforward in the art to determine whether or not a protein binds to a polynucleotide. The protein typically interacts with and modifies at least one property of the polynucleotide. The protein may modify the polynucleotide by cleaving it to form individual nucleotides or shorter chains of nucleotides, such as di- or trinucleotides. The moiety may modify the polynucleotide by orienting it or moving it to a specific position, i.e. controlling its movement.

The polynucleotide binding protein is preferably a polynucleotide handling enzyme. A polynucleotide handling enzyme is a polypeptide that is capable of interacting with and

modifying at least one property of a polynucleotide. The enzyme may modify the polynucleotide by cleaving it to form individual nucleotides or shorter chains of nucleotides, such as di- or trinucleotides. The enzyme may modify the polynucleotide by orienting it or moving it to a specific position. The polynucleotide handling enzyme does not need to display enzymatic activity as long as it is capable of binding the target sequence and controlling its movement through the pore. For instance, the enzyme may be modified to remove its enzymatic activity or may be used under conditions which prevent it from acting as an enzyme. Such conditions are discussed in more detail below.

The polynucleotide handling enzyme is preferably derived from a nucleolytic enzyme. The polynucleotide handling enzyme used in the construct of the enzyme is more preferably derived from a member of any of the Enzyme Classification (EC) groups 3.1.11, 3.1.13, 3.1.14, 3.1.15, 3.1.16, 3.1.21, 3.1.22, 3.1.25, 3.1.26, 3.1.27, 3.1.30 and 3.1.31. The enzyme may be any of those disclosed in International Application No. PCT/GB10/000133 (published as WO 2010/086603).

Preferred enzymes are polymerases, exonucleases, helicases and topoisomerases, such as gyrases. Suitable enzymes include, but are not limited to, exonuclease I from E. coli (SEQ ID NO: 11), exonuclease III enzyme from E. coli (SEQ ID NO: 13), RecJ from T. thermophilus (SEQ ID NO: 15) and bacteriophage lambda exonuclease (SEQ ID NO: 17) and variants thereof. Three subunits comprising the sequence shown in SEQ ID NO: 15 or a variant thereof interact to form a trimer exonuclease. The enzyme is preferably Phi29 DNA polymerase (SEQ ID NO: 9) or a variant thereof. The topoisomerase is preferably a member of any of the Moiety Classification (EC) groups 5.99.1.2 and 5.99.1.3.

The enzyme is most preferably derived from a helicase, such as Hel308 Mbu (SEQ ID NO: 18), Hel308 Csy (SEQ ID NO: 19), Hel308 Tga (SEQ ID NO: 20); Hel308 Mhu (SEQ ID NO: 21), TraI Eco (SEQ ID NO: 22), XPD Mbu (SEQ ID NO: 23) or a variant thereof.

A variant of SEQ ID NOs: 9, 11, 13, 15, 17, 18, 19, 20, 21, 22 or 23 is an enzyme that has an amino acid sequence which varies from that of SEQ ID NO: 9, 11, 13, 15, 17, 18, 19, 20, 21, 22 or 23 and which retains polynucleotide binding ability. This can be measured using any method known in the art. For instance, the variant can be contacted with a polynucleotide and its ability to bind to and move along the polynucleotide can be measured. The variant may include modifications that facilitate binding of the polynucleotide and/or facilitate its activity at high salt concentrations and/or room temperature.

Over the entire length of the amino acid sequence of SEQ ID NO: 9, 11, 13, 15, 17, 18, 19, 20, 21, 22 or 23, a variant will preferably be at least 50% homologous to that sequence

based on amino acid identity. More preferably, the variant polypeptide may be at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% and more preferably at least 95%, 97% or 99% homologous based on amino acid identity to the amino acid sequence of SEQ ID NO: 9, 11, 13, 15, 17, 18, 19, 20, 21, 22 or 23 over the entire sequence. There may be at least 80%, for example at least 85%, 90% or 95%, amino acid identity over a stretch of 200 or more, for example 230, 250, 270, 280, 300, 400, 500, 600, 700, 800, 900 or 1000 or more, contiguous amino acids ("hard homology"). Homology is determined as described above. The variant may differ from the wild-type sequence in any of the ways discussed above with reference to SEQ ID NO: 2 and 4 above. The enzyme may be covalently attached to the pore. Any method may be used to covalently attach the enzyme to the pore.There are two main strategies for sequencing polynucleotides using nanopores, namely strand sequencing and exonuclease sequencing. The method of the invention may concern either strand sequencing or exonuclease sequencing.

In strand sequencing, the DNA is translocated through the nanopore either with or against an applied potential. Exonucleases that act progressively or processively on double stranded DNA can be used on the *cis* side of the pore to feed the remaining single strand through under an applied potential or the *trans* side under a reverse potential. Likewise, a helicase that unwinds the double stranded DNA can also be used in a similar manner. A polymerase may also be used. There are also possibilities for sequencing applications that require strand translocation against an applied potential, but the DNA must be first "caught" by the enzyme under a reverse or no potential. With the potential then switched back following binding the strand will pass *cis* to *trans* through the pore and be held in an extended conformation by the current flow. The single strand DNA exonucleases or single strand DNA dependent polymerases can act as molecular motors to pull the recently translocated single strand back through the pore in a controlled stepwise manner, *trans* to *cis*, against the applied potential.

In one embodiment, the method of characterising a target polynucleotide involves contacting the target sequence with a pore and a helicase enzyme. Any helicase may be used in the method. Helicases may work in two modes with respect to the pore. First, the method is preferably carried out using a helicase such that it moves the target sequence through the pore with the field resulting from the applied voltage. In this mode the 5' end of the DNA is first captured in the pore, and the enzyme moves the DNA into the pore such that the target sequence is passed through the pore with the field until it finally translocates through to the trans side of the bilayer. Alternatively, the method is preferably carried out such that a

helicase enzyme moves the target sequence through the pore against the field resulting from the applied voltage. In this mode the 3' end of the DNA is first captured in the pore, and the enzyme moves the DNA through the pore such that the target sequence is pulled out of the pore against the applied field until finally ejected back to the cis side of the bilayer.

In exonuclease sequencing, an exonuclease releases individual nucleotides from one end of the target polynucleotie and these individual nucleotides are identified as discussed below. In another embodiment, the method of characterising a target polynucleotide involves contacting the target sequence with a pore and an exonuclease enzyme. Any of the exonuclease enzymes discussed above may be used in the method. The enzyme may be covalently attached to the pore as discussed above.

Exonucleases are enzymes that typically latch onto one end of a polynucleotide and digest the sequence one nucleotide at a time from that end. The exonuclease can digest the polynucleotide in the 5′ to 3′ direction or 3′ to 5′ direction. The end of the polynucleotide to which the exonuclease binds is typically determined through the choice of enzyme used and/or using methods known in the art. Hydroxyl groups or cap structures at either end of the polynucleotide may typically be used to prevent or facilitate the binding of the exonuclease to a particular end of the polynucleotide.

The method involves contacting the polynucleotide with the exonuclease so that the nucleotides are digested from the end of the polynucleotide at a rate that allows characterisation or identification of a proportion of nucleotides as discussed above. Methods for doing this are well known in the art. For example, Edman degradation is used to successively digest single amino acids from the end of polypeptide such that they may be identified using High Performance Liquid Chromatography (HPLC). A homologous method may be used in the present invention.

The rate at which the exonuclease functions is typically slower than the optimal rate of a wild-type exonuclease. A suitable rate of activity of the exonuclease in the method of the invention involves digestion of from 0.5 to 1000 nucleotides per second, from 0.6 to 500 nucleotides per second, 0.7 to 200 nucleotides per second, from 0.8 to 100 nucleotides per second, from 0.9 to 50 nucleotides per second or 1 to 20 or 10 nucleotides per second. The rate is preferably 1, 10, 100, 500 or 1000 nucleotides per second. A suitable rate of exonuclease activity can be achieved in various ways. For example, variant exonucleases with a reduced optimal rate of activity may be used in accordance with the invention.

The method of the invention involves measuring one or more characteristics of the target analyte, such as a target polynucleotide. The method may involve measuring two,

three, four or five or more characteristics of the target analyte, such as a target polynucleotide. For target polynucleotides, the one or more characteristics are preferably selected from (i) the length of the target polynucleotide, (ii) the identity of the target polynucleotide, (iii) the sequence of the target polynucleotide, (iv) the secondary structure of the target polynucleotide and (v) whether or not the target polynucleotide is modified. Any combination of (i) to (v) may be measured in accordance with the invention.

For (i), the length of the polynucleotide may be measured using the number of interactions between the target polynucleotide and the pore.

For (ii), the identity of the polynucleotide may be measured in a number of ways. The identity of the polynucleotide may be measured in conjunction with measurement of the sequence of the target polynucleotide or without measurement of the sequence of the target polynucleotide. The former is straightforward; the polynucleotide is sequenced and thereby identified. The latter may be done in several ways. For instance, the presence of a particular motif in the polynucleotide may be measured (without measuring the remaining sequence of the polynucleotide). Alternatively, the measurement of a particular electrical and/or optical signal in the method may identify the target polynucleotide as coming from a particular source.

For (iii), the sequence of the polynucleotide can be determined as described previously. Suitable sequencing methods, particularly those using electrical measurements, are described in Stoddart D et al., Proc Natl Acad Sci, 12;106(19):7702-7, Lieberman KR et al, J Am Chem Soc. 2010;132(50):17961-72, and International Application WO 2000/28312.

For (iv), the secondary structure may be measured in a variety of ways. For instance, if the method involves an electrical measurement, the secondary structure may be measured using a change in dwell time or a change in current flowing through the pore. This allows regions of single-stranded and double-stranded polynucleotide to be distinguished.

For (v), the presence or absence of any modification may be measured. The method preferably comprises determining whether or not the target polynucleotide is modified by methylation, by oxidation, by damage, with one or more proteins or with one or more labels, tags or spacers. Specific modifications will result in specific interactions with the pore which can be measured using the methods described below. For instance, methylcyotsine may be distinguished from cytosine on the basis of the current flowing through the pore during its interation with each nucleotide.

The invention also provides a method of estimating the sequence of a target polynucleotide. The invention further provides a method of sequencing a target polynucleotide.

A variety of different types of measurements may be made. This includes without limitation: electrical measurements and optical measurements. Possible electrical measurements include: current measurements, impedance measurements, tunnelling measurements (Ivanov AP et al., Nano Lett. 2011 Jan 12;11(1):279-85), and FET measurements (International Application WO 2005/124888). Optical measurements may be combined 10 with electrical measurements (Soni GV et al., Rev Sci Instrum. 2010 Jan;81(1):014301). The measurement may be a transmembrane current measurement such as measurement of ionic current flowing through the pore.

Electrical measurements may be made using standard single channel recording equipment as describe in Stoddart D et al., Proc Natl Acad Sci, 12;106(19):7702-7, Lieberman KR et al, J Am Chem Soc. 2010;132(50):17961-72, and International Application WO-2000/28312. Alternatively, electrical measurements may be made using a multi-channel system, for example as described in International Application WO-2009/077734 and International Application WO-2011/067559.

In a preferred embodiment, step (b) comprises measuring the current passing through the pore as the analyte moves with respect to the pore wherein the current is indicative of one or more characteristics of the target analyte and thereby characterising the target analyte. In a more preferred embodiment, the target analyte is a target polynucleotide and the method comprises (a) contacting the target polynucleotide with a transmembrane pore present in a membrane of the system of the invention and a polynucleotide binding protein such that the protein controls the movement of the target polynucleotide through the pore and (b) measuring the current passing through the pore as the polynucleotide moves with respect to the pore wherein the current is indicative of one or more characteristics of the target polynucleotide and thereby characterising the target polynucleotide.

The methods may be carried out using any apparatus that is suitable for investigating a membrane/pore system in which a pore is inserted into a membrane of the system of the invention.

The methods may involve measuring the current passing through the pore as the analyte, such as a target polynucleotide, moves with respect to the pore. Therefore the apparatus may also comprise an electrical circuit capable of applying a potential and measuring an electrical signal across the membrane and pore. The methods may be carried

out using a patch clamp or a voltage clamp. The methods preferably involve the use of a voltage clamp.

The methods of the invention may involve the measuring of a current passing through the pore as the analyte, such as a target polynucleotide, moves with respect to the pore. Suitable conditions for measuring ionic currents through transmembrane protein pores are known in the art and disclosed in the Example. The method is typically carried out with a voltage applied across the membrane and pore. The voltage used is typically from +2 V to -2 V, typically -400 mV to +400mV. The voltage used is preferably in a range having a lower limit selected from -400 mV, -300 mV, -200 mV, -150 mV, -100 mV, -50 mV, -20mV and 0 mV and an upper limit independently selected from +10 mV, + 20 mV, +50 mV, +100 mV, +150 mV, +200 mV, +300 mV and +400 mV. The voltage used is more preferably in the range 100 mV to 240mV and most preferably in the range of 120 mV to 220 mV. It is possible to increase discrimination between different nucleotides by a pore by using an increased applied potential.

The methods are typically carried out in the presence of any charge carriers, such as metal salts, for example alkali metal salt, halide salts, for example chloride salts, such as alkali metal chloride salt. Charge carriers may include ionic liquids or organic salts, for example tetramethyl ammonium chloride, trimethylphenyl ammonium chloride, phenyltrimethyl ammonium chloride, or 1-ethyl-3-methyl imidazolium chloride. In the exemplary apparatus discussed above, the salt is present in the aqueous solution in the chamber. Potassium chloride (KCl), sodium chloride (NaCl) or caesium chloride (CsCl) is typically used. KCl is preferred. The salt concentration may be at saturation. The salt concentration may be 3M or lower and is typically from 0.1 to 2.5 M, from 0.3 to 1.9 M, from 0.5 to 1.8 M, from 0.7 to 1.7 M, from 0.9 to 1.6 M or from 1 M to 1.4 M. The salt concentration is preferably from 150 mM to 1 M. The method is preferably carried out using a salt concentration of at least 0.3 M, such as at least 0.4 M, at least 0.5 M, at least 0.6 M, at least 0.8 M, at least 1.0 M, at least 1.5 M, at least 2.0 M, at least 2.5 M or at least 3.0 M. High salt concentrations provide a high signal to noise ratio and allow for currents indicative of the presence of a nucleotide to be identified against the background of normal current fluctuations.

The methods are typically carried out in the presence of a buffer. In the exemplary apparatus discussed above, the buffer is present in the aqueous solution in the chamber. Any buffer may be used in the method of the invention. Typically, the buffer is HEPES. Another suitable buffer is Tris-HCl buffer. The methods are typically carried out at a pH of from 4.0

to 12.0, from 4.5 to 10.0, from 5.0 to 9.0, from 5.5 to 8.8, from 6.0 to 8.7 or from 7.0 to 8.8 or 7.5 to 8.5. The pH used is preferably about 7.5.

The methods may be carried out at from 0 °C to 100 °C, from 15 °C to 95 °C, from 16 °C to 90 °C, from 17 °C to 85 °C, from 18 °C to 80 °C, 19 °C to 70 °C, or from 20 °C to 60 °C. The methods are typically carried out at room temperature. The methods are optionally carried out at a temperature that supports enzyme function, such as about 37 °C.

The method is typically carried out in the presence of free nucleotides or free nucleotide analogues and an enzyme cofactor that facilitate the action of the polynucleotide binding protein, such as a helicase or an exonuclease. The free nucleotides may be one or more of any of the individual nucleotides discussed above. The free nucleotides include, but are not limited to, adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), guanosine monophosphate (GMP), guanosine diphosphate (GDP), guanosine triphosphate (GTP), thymidine monophosphate (TMP), thymidine diphosphate (TDP), thymidine triphosphate (TTP), uridine monophosphate (UMP), uridine diphosphate (UDP), uridine triphosphate (UTP), cytidine monophosphate (CMP), cytidine diphosphate (CDP), cytidine triphosphate (CTP), cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), deoxyadenosine monophosphate (dAMP), deoxyadenosine diphosphate (dADP), deoxyadenosine triphosphate (dATP), deoxyguanosine monophosphate (dGMP), deoxyguanosine diphosphate (dGDP), deoxyguanosine triphosphate (dGTP), deoxythymidine monophosphate (dTMP), deoxythymidine diphosphate (dTDP), deoxythymidine triphosphate (dTTP), deoxyuridine monophosphate (dUMP), deoxyuridine diphosphate (dUDP), deoxyuridine triphosphate (dUTP), deoxycytidine monophosphate (dCMP), deoxycytidine diphosphate (dCDP) and deoxycytidine triphosphate (dCTP). The free nucleotides are preferably selected from AMP, TMP, GMP, CMP, UMP, dAMP, dTMP, dGMP or dCMP. The free nucleotides are preferably adenosine triphosphate (ATP). The enzyme cofactor is a factor that allows the helicase to function. The enzyme cofactor is preferably a divalent metal cation. The divalent metal cation is preferably Mg^{2+}, Mn^{2+}, Ca^{2+} or Co^{2+}. The enzyme cofactor is most preferably Mg^{2+}.

Methods of forming sensors

The invention also provides a method of forming a sensor for characterising a target polynucleotide. The method comprises forming a complex between (a) a pore present in a membrane of the system of the invention and (b) a polynucleotide binding protein, such as a helicase or an exonuclease. The complex may be formed by contacting the pore and the

protein in the presence of the target polynucleotide and then applying a potential across the pore. The applied potential may be a chemical potential or a voltage potential as described above. Alternatively, the complex may be formed by covalently attaching the pore to the protein. Methods for covalent attachment are known in the art and disclosed, for example, in International Application Nos. PCT/GB09/001679 (published as WO 2010/004265) and PCT/GB10/000133 (published as WO 2010/086603). The complex is a sensor for characterising the target polynucleotide. The method preferably comprises forming a complex between the pore and a helicase. Any of the embodiments discussed above equally apply to this method.

The invention also provides a sensor for characterising a target polynucleotide. The sensor comprises a complex between (a) a pore present in a membrane of the system of the invention and (b) a polynucleotide binding protein. Any of the embodiments discussed above equally apply to the sensor of the invention.

Kits

The present invention also provides a kit for characterising, such as sequencing, a target polynucleotide. The kit comprises (a) a pore present in a membrane of the system of the invention and (b) a polynucleotide binding protein, such as a helicase or an exonuclease. Any of the embodiments discussed above equally applicable to the kits of the invention.

The kits of the invention may additionally comprise one or more other reagents or instruments which enable any of the embodiments mentioned above to be carried out. Such reagents or instruments include one or more of the following: suitable buffer(s) (aqueous solutions), means to obtain a sample from a subject (such as a vessel or an instrument comprising a needle), means to amplify and/or express polynucleotide sequences or voltage or patch clamp apparatus. Reagents may be present in the kit in a dry state such that a fluid sample resuspends the reagents. The kit may also, optionally, comprise instructions to enable the kit to be used in the method of the invention or details regarding which patients the method may be used for. The kit may, optionally, comprise nucleotides.

Apparatus

The invention also provides an apparatus for characterising, such as sequencing, target polynucleotides in a sample. The apparatus may comprise (a) a plurality of pores present in a plurality of membranes of one or more systems of the invention and (b) a plurality of

polynucleotide binding proteins, such as helicases or exonucleases. The apparatus may be any conventional apparatus for analyte analysis, such as an array or a chip.

The apparatus preferably comprises:

a sensor device that is capable of supporting the plurality of pores and membranes and being operable to perform polynucleotide characterising or sequencing using the pores and proteins;

- at least one reservoir for holding material for performing the characterising or sequencing;
- a fluidics system configured to controllably supply material from the at least one reservoir to the sensor device; and
- a plurality of containers for receiving respective samples, the fluidics system being configured to supply the samples selectively from the containers to the sensor device.

The apparatus may be any of those described in International Application No. PCT/GB10/000789 (published as WO 2010/122293), International Application No. PCT/GB10/002206 (not yet published) or International Application No. PCT/US99/25679 (published as WO 00/28312).

The invention will be further described in the Examples which follow.

EXAMPLES

Example 1

This example describes the method used to produce the triblock co-polymer droplets which are used to fill the interconnecting compartments on the microfluidic chips.

Materials and Methods

The T-junction chips are prepared for droplet generation by affixing nanoport assemblies (Upchurch Scientific) as fluidic interfaces.

The droplet generation mechanism in a T-junction is well documented in the literature [Garstecki et al., Lab Chip, 2006, 6, 437-446 and Thorsen et al., Physical Reviw Letters, 86, 18, 4163-4166]. Taking into account the fluid viscosities of the reagents involved the chosen T-junction geometry was 50 μm channel width for both cases (oil and buffer).

1.1 - Droplet reagents

In order to make aqueous phase droplets in oil, buffer is used as the disperse phase, while a silicon oil (e.g. AR20), is used as the continuous phase. Both buffer and triblock co-polymer-containing oil are prepared as described below.

A solution of buffer (buffer 1) was prepared by adding 298mg of KCl (99.99% Purity, Sigma) to 10 mL of degassed DI water. To this solution 30.35mg of 2-Amino-2-(hydroxymethyl)-1,3-propanediol (99.9%, Sigma) was added. The solution was buffered to pH 8 using small quantities of HCl and NaOH. 316.5mg of K2[Fe(CN)6] (99.9%, Sigma) and 82.3mg of K3[Fe(CN)6] (99.9%, Sigma) was added to the solution and stirred until dissolved.

Oil-triblock co-polymer solution was prepared by adding 20 mg of polymer (6-33-6, PMOXA-PDMS-PMOXA, PolymerSource) to 1 mL of AR20 (99%, Sigma). The polymer was left stirring in the oil for 24 hrs until all of the polymer had dissolved.

1.2 - Droplet generation setup

A schematic for the droplet generation setup can be seen in Fig. 1. This setup consists of two syringe pumps (Elite, Harvard Apparatus), two gastight syringes (Hamilton), peak tubing (Upchurch Scientific), and a custom made T-junction microfluidic chip. Once the syringes are loaded with oil and buffer and mounted on the syringe pumps, the peak tubing is used to establish the fluidic connections to the ports on the chip. The oil syringe should be connected to the continuous phase channel input while the buffer should be connected to the disperse phase channel input.

Both syringe pumps were set to infuse at a flow rate of 10 μL/min, which produced an average droplet size (diameter) of 129.46 μm, with a standard deviation of 10.87 μm. The droplets were then collected in a vial.

Example 2

This example describes the method used to produce droplet-interface-bilayers (DIBs) using a number of different tri-block co-polymers in different oils (experimental set-up shown in Fig. 3). The ability to form bilayers and to allow insertion of biological nanopores (such as mutants of MspA) was also investigated.

Materials and Methods

Experiments 2.1, 2.3 and 2.4 were carried out on the below combinations of tri-block co-polymer and oil.

1 – 6-33-6 (PMOXA-PDMS-PMOXA) PolymerSource (20 mg/mL) in AR20 oil (polyphenyl-methylsiloxane, Sigma Aldrich).

2 – 6-33-6 (PMOXA-PDMS-PMOXA) PolymerSource (20 mg/mL) in PDMS-OH 65cSt oil (poly(dimethylsiloxane), hydroxyl terminated, Sigma Aldrich).

3 – 6-45PE-6 (PMOXA-PE-PMOXA, where PE = a polyethylene hydrocarbon chain approximately 45 carbon atoms in length.) PolymerSource (20 mg/mL) in hexadecane (99.9%, Sigma Aldrich).

4 – 6-32-6 (PMOXA-PDMS-PMOXA) HighForce (20 mg/mL) in AR20 oil (polyphenyl-methylsiloxane, Sigma Aldrich).

2.1 – Droplet stability experiments

Droplet stability was measured off-line by preparing solutions of buffer and triblock ABA polymer in various oils. A small 0.5 cm^2 tray was prepared using polycarbonate and a glass slide (Fig. 2). The tray was filled with oil. To the oil, 1μL buffer droplets were added and monitored over 24hrs. Droplets that exhibited only a small degree of merging were progressed to electrical DIBs testing.

2.2 - Experimental set-up

The experimental apparatus was set-up as shown in Fig. 3. A 700B axopatch was connected inside a shielded box containing two micro-manipulators. The entire faraday cage was placed on an inverted microscope (Nikon) such that it was possible to view the manipulation of the droplets from underneath. This allowed the droplets to be moved without opening the Faraday cage.

Within the Faraday cage, the electrodes of the 700B axopatch were connected via pure gold (Au) wire

The Au was prepared for use in the droplet setup by flaming the end such that the wire formed a small gold bead. The Au wire was cleaned by emersion in conc.HNO_3 for 30 s, and washed thoroughly with DI water. The ball-ended wire was then repeatedly moved through a liquid agarose solution prepared from the buffer (5% wt low-melt agarose, Lonza / Buffer 400 mM KCl, 75 mM K2[Fe(CN)6] (99.9%, Sigma) and 25 mM K3[Fe(CN)6] (99.9%, Sigma), 10mM Tris). Once a small bead had formed on the end the agarose was

allowed to cool, and the wire was stored in an excess of buffer solution in order to come to equilibrium.

The droplet chamber was mounted on the stage within the Faraday cage, and the electrodes were mounted such that both fell within the central section of the chamber. The manipulators were situated such that a full range of movement in X and Y directions were achievable by both electrodes over the area of the chamber. The chamber was then filled to the brim with the AR20 tri-block co-polymer solution and allowed to stand for a few minutes. 1μL of buffer was pipetted directly onto each of the agarose tipped Au wires and both electrodes were moved directly under the AR20/triblock co-polymer solution. The droplets were left under the solution for 30 s before movement.

2.3 – Membrane formation

To form a membrane with the droplet pair, a waveform of ±20 mV was applied to the electrodes in addition to a bias voltage of 180 mV. The current response was monitored as the indicator of the formation of a capacitive membrane (see Fig. 5 for a sample trace showing the increase in capacitance over time). The droplets were carefully brought together such that contact between the two buffer volumes was made (see Fig. 4 (B)). The droplets were left in this state until a membrane was formed (see Fig. 5). In situations where the membrane growth was very slow, the droplets were moved in the XY direction, which forced exclusion of the AR20/triblock co-polymer between the droplets and facilitated membrane growth.

2.4 – Nanopore insertion experiments

In order to insert trans-membrane pores across the membrane, a 0.0005 mg/ml solution of MspA-(B2C) (SEQ ID NO: 25, which is a variant of SEQ ID NO: 2 with the following mutations G75S/G77S/L88N/Q126R) was added to the buffer that formed the anolyte. Insertion of the pore was observed by an instantaneous increase in current (See Fig. 6). This was performed in the absence of the waveform, but under the applied bias potential.

Results

The different tri-block co-polymer and oil combinations that were investigated are shown in table 4 below.

Table 4

Tri-Block Co-Polymer	**Oil**	**Off-line Stability Test**	**Membrane Formation**	**MspA-(B2C) Pore Insertion**
6-33-6 PolymerSource	AR20	stable droplets formed	capacitive membrane growth observed	pores inserted
6-33-6 PolymerSource	PDMS-OH 65cSt	stable droplets formed	capacitive membrane growth observed	pores inserted
6-45PE-6 PolymerSource	C16	stable droplets formed	capacitive membrane growth observed	pores inserted
6-32-6 HighForce	AR20	stable droplets formed	capacitive membrane growth observed	pores inserted

Capacitive membrane growth and pore insertion was observed for all of the tri-block co-polymer/oils tested. Fig. 5 and 6 show membrane growth and MspA-(B2C) (SEQ ID NO: 25, which is a variant of SEQ ID NO: 2 with the following mutations G75S/G77S/L88N/Q126R) pore insertion for the 6-33-6 PolymerSource tri-block co-polymer used with AR20 silicone oil. Fig. 7 shows membrane growth and pore insertion for the 6-45PE-6 PolymerSource used with hexadecane as an example of a triblock co-polymer which does not have the PMOXA central core structure.

The Oxford Nanopore Technologies Limited reference for this application is ONT IP 039.

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Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method of forming a membrane between a first volume of polar medium and a second volume of polar medium, which method comprises:

(a) providing a first volume comprising polar medium and a second volume comprising polar medium which are separated from one another by an apolar medium,

wherein at least one of said first and second volumes comprises a layer comprising amphipathic molecules at the interface between the polar medium and the apolar medium,

wherein each of the amphipathic molecules comprises a first outer hydrophilic group, a hydrophobic core group, and a second outer hydrophilic group, wherein each of the first and second outer hydrophilic groups is linked to the hydrophobic core group; and

(b) causing the first and second volumes to come into contact with one another to form a membrane comprising said amphipathic molecules between the first and second volumes of polar medium.

2. A method according to claim 1 wherein each of the first and second volumes comprises a layer comprising the amphipathic molecules at the interface between the polar medium and the apolar medium.

3. A method according to claim 1 or claim 2 wherein the first and second outer hydrophilic groups are independently linked to different atoms of the hydrophobic core group.

4. A method according to any one of claims 1 to 3 wherein the first and second outer hydrophilic groups are linked to opposite ends of the hydrophobic core group.

5. A method according to any one of the preceding claims wherein each of the amphipathic molecules is a copolymer comprising at least three polymer segments, wherein the hydrophobic core group is an inner hydrophobic polymer segment, B, and the first and second outer hydrophilic groups are first and second outer hydrophilic polymer segments, A_1 and A_2.

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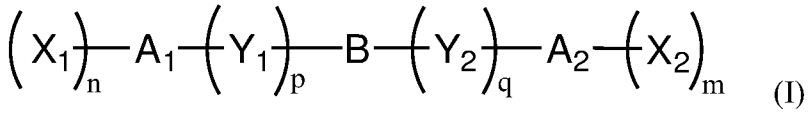
6. A method according to claim 5 wherein the copolymer has a linear or graft structure, and wherein the first and second outer hydrophilic polymer segments, A_1 and A_2, are pendant from the inner hydrophobic polymer segment, B.

7. A method according to claim 5 or claim 6 wherein the copolymer is a block copolymer of formula (I)



(I)

wherein:

A_1 is said first outer hydrophilic polymer segment;

B is said inner hydrophobic polymer segment;

A_2 is said second outer hydrophilic polymer segment;

X_1, Y_1, Y_2 and X_2 are additional polymer segments; and

n, p, q and m are independently either 0 or 1.

8. A method according to any one of claims 5 to 7, wherein the inner hydrophobic polymer segment B comprises a polysiloxane or a polyalkene.

9. A method according to any one of claims 5 to 8, wherein the first outer hydrophilic polymer segment A_1 and the second outer hydrophilic polymer segment A_2, which may be the same or different, comprise poly(2-methyloxazoline).

10. A method according to any one of the preceding claims wherein each of the amphipathic molecules is poly(2-methyloxazoline)-block-poly(dimethylsiloxane)-block-poly(2-methyloxazoline) (PMOXA-PDMS-PMOXA).

11. A method according to any one of the preceding claims wherein the first volume comprising polar medium is a droplet or bead.

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12. A method according to any one of the preceding claims which comprises:

(a) providing at least three volumes comprising polar medium which are separated from one another by an apolar medium, wherein at least one of, or each of, said volumes comprises a layer comprising said amphipathic molecules at the interface between the polar medium and the apolar medium; and

(b) causing each of said volumes to come into contact with another of said volumes to form a membrane comprising said amphipathic molecules between the volumes of polar medium.

13. A method according to claim 12 wherein each of said volumes is a droplet or bead and step (b) produces a chain or network of the droplets or beads.

14. A method according to any one of the preceding claims wherein the membrane comprising said amphipathic molecules further comprises a membrane protein.

15. A method according to claim 14 wherein the membrane protein is an ion channel or pore.

16. A method according to any one of the preceding claims, further comprising bringing electrodes into electrical contact with the volumes of polar medium and taking an electrical measurement using the electrodes.

17. A system comprising

a first volume of a polar medium;

a second volume of a polar medium; and

a membrane between the first and second volumes of polar medium, which membrane comprises amphipathic molecules,

wherein each of the amphipathic molecules comprises a first outer hydrophilic group, a hydrophobic core group, and a second outer hydrophilic group, wherein each of the first and second outer hydrophilic groups is linked to the hydrophobic core group,

and wherein the first volume of polar medium is within an apolar medium.

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18. A system according to claim 17 which comprises one or more further volumes of polar medium and one or more further membranes comprising said amphipathic molecules, wherein each further volume of polar medium is separated from another of the volumes of polar medium by a said further membrane.

19. A volume comprising polar medium, which volume is disposed within an apolar medium, and which volume has a layer comprising amphipathic molecules around a surface thereof, between the polar medium and the apolar medium, wherein each of the amphipathic molecules comprises a first outer hydrophilic group, a hydrophobic core group, and a second outer hydrophilic group, wherein each of the first and second outer hydrophilic groups is linked to the hydrophobic core group, and wherein each of the amphipathic molecules is a copolymer comprising at least three polymer segments, wherein the hydrophobic core group is an inner hydrophobic polymer segment, B, and the first and second outer hydrophilic groups are first and second outer hydrophilic polymer segments, A_1 and A_2.

20. A process for producing a volume comprising polar medium, which volume is disposed within an apolar medium, and which volume has a layer comprising amphipathic molecules around a surface thereof, between the polar medium and the apolar medium, wherein each of the amphipathic molecules comprises a first outer hydrophilic group, a hydrophobic core group, and a second outer hydrophilic group, wherein each of the first and second outer hydrophilic groups is linked to the hydrophobic core group, and wherein each of the amphipathic molecules is a copolymer comprising at least three polymer segments, wherein the hydrophobic core group is an inner hydrophobic polymer segment, B, and the first and second outer hydrophilic groups are first and second outer hydrophilic polymer segments, A_1 and A_2,

which process comprises:

(i) introducing a volume of a polar medium into an apolar medium;

(ii) providing the amphipathic molecules, in the apolar medium or the polar medium or both, either before or after (i); and

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(iii) leaving the volume of polar medium for a time sufficient for the layer comprising the amphipathic molecules to form at the interface between the polar medium and the apolar medium.

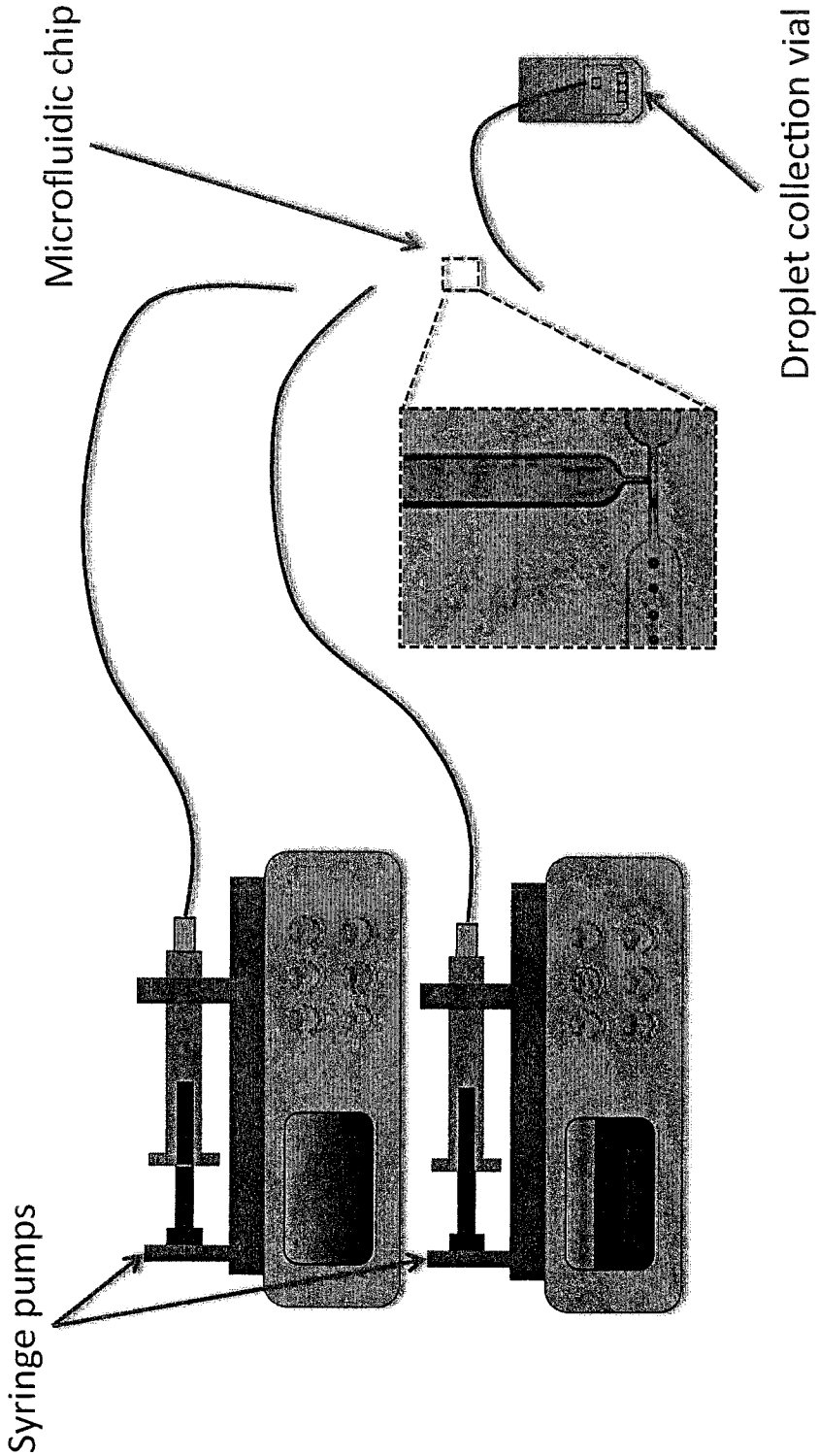


Fig. 1

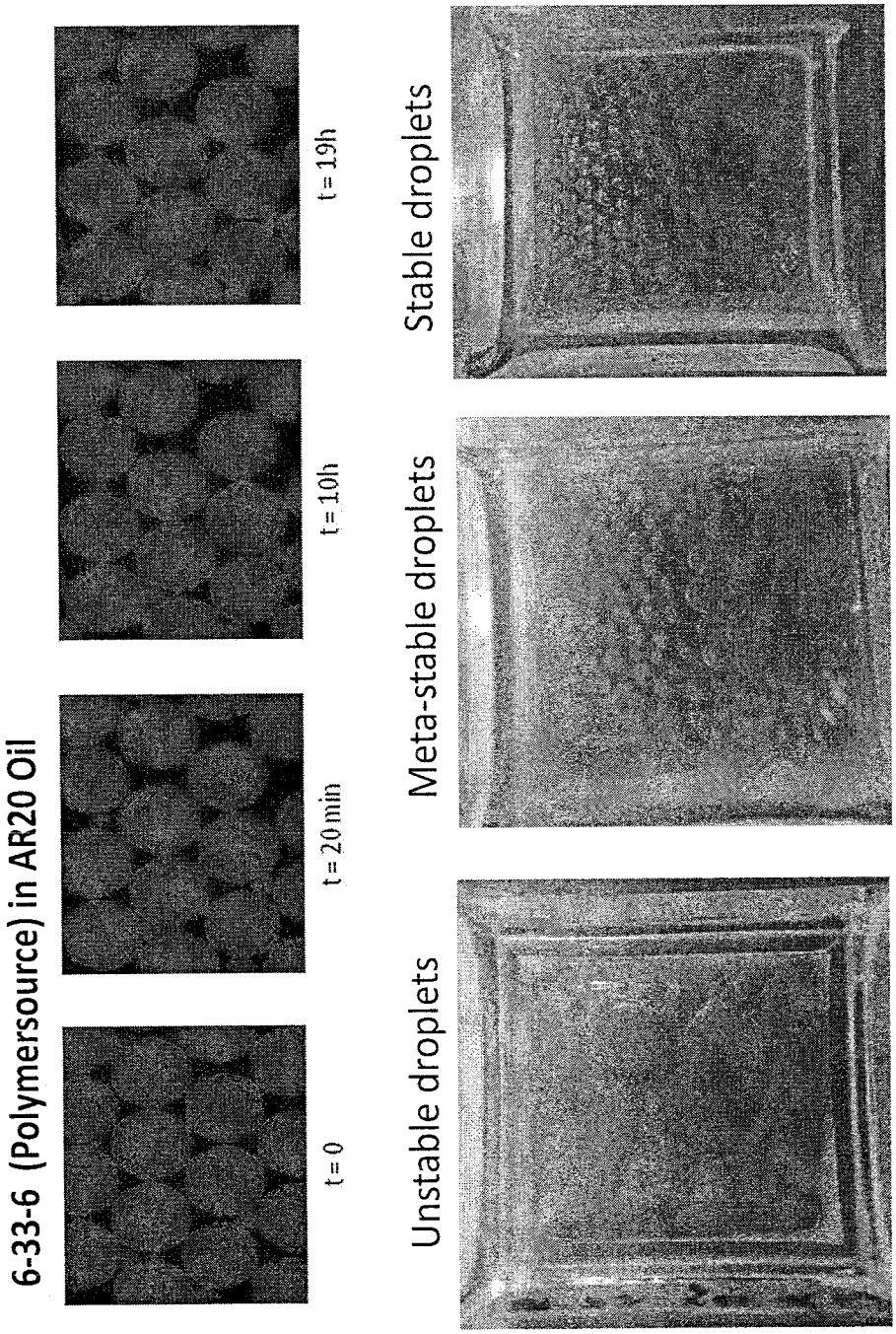

6-33-6 (Polymersource) in AR20 Oil
t = 0
t = 20 min
t = 10h
t = 19h
Unstable droplets
Meta-stable droplets
Stable droplets

Fig. 2

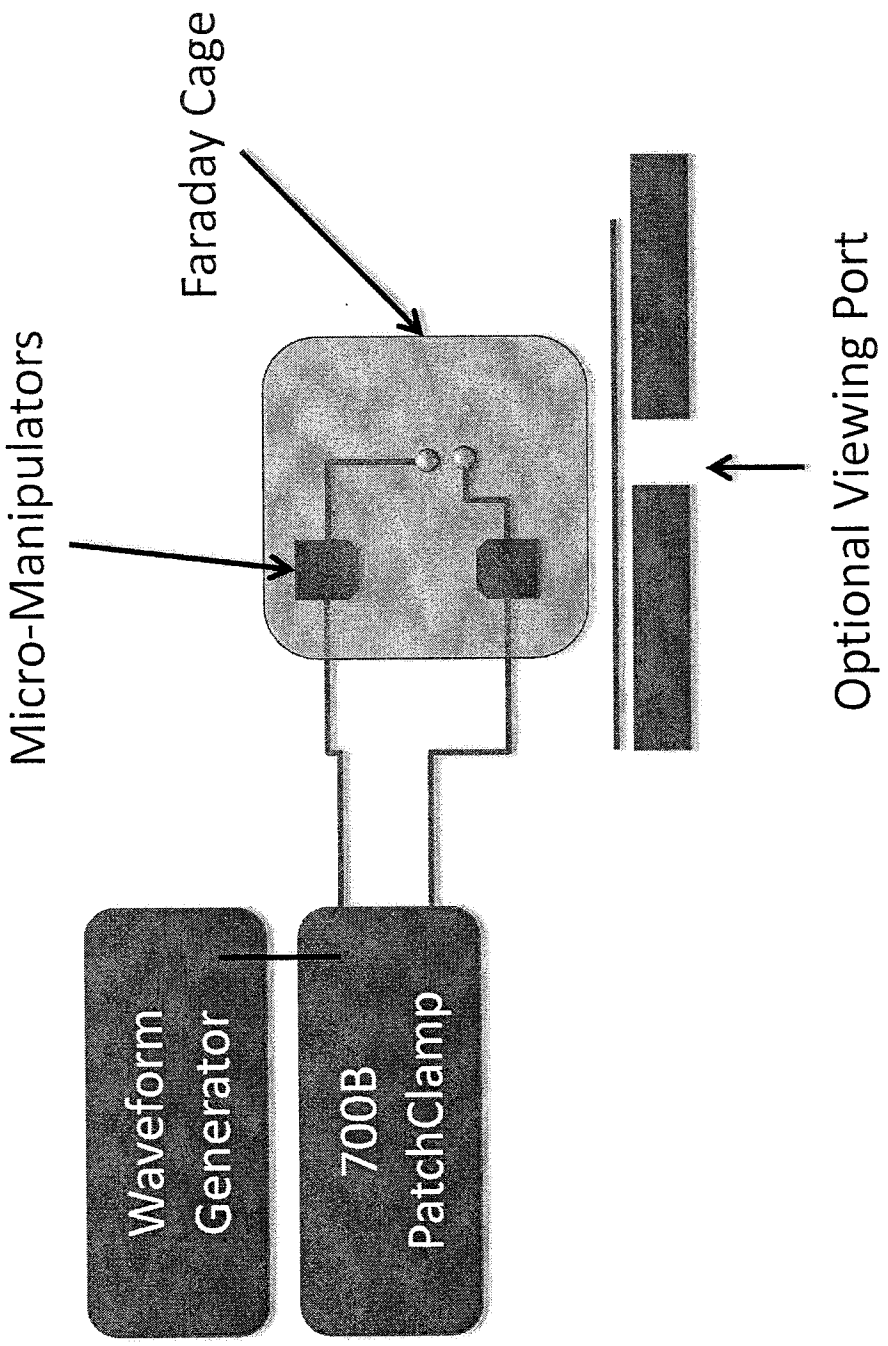

Micro-Manipulators
Faraday Cage
Optional Viewing Port
Waveform Generator
700B PatchClamp

Fig. 3

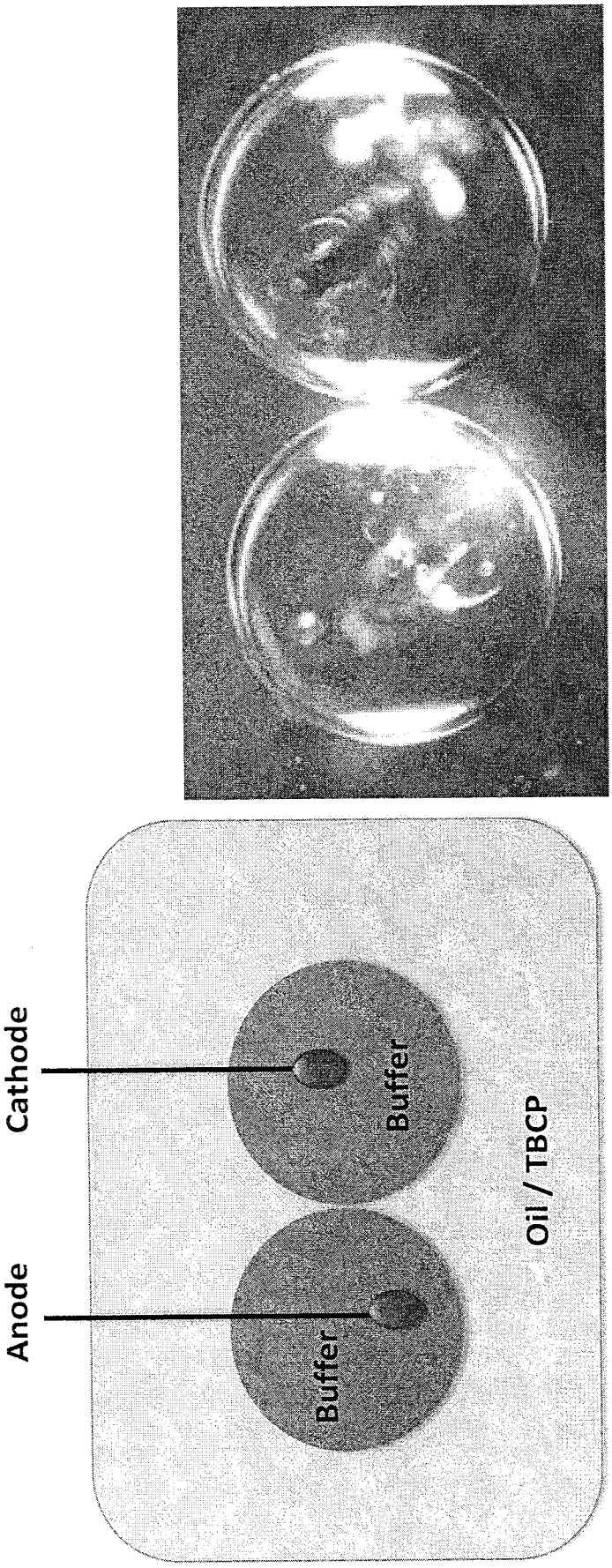


Fig. 4

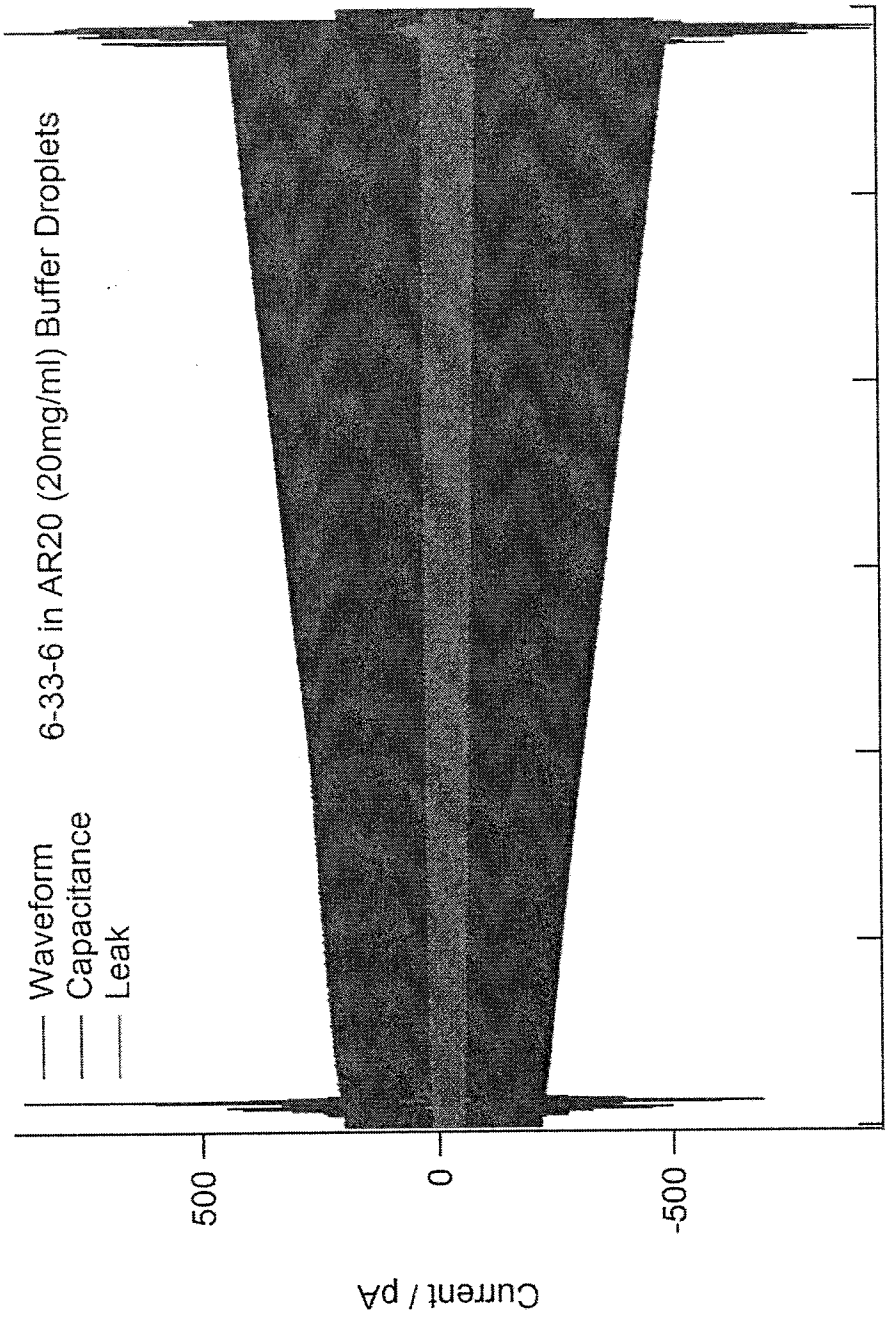

6-33-6 in AR20 (20mg/ml) Buffer Droplets
Waveform
Capacitance
Leak
500
0
-500
Current / pA

Fig. 5

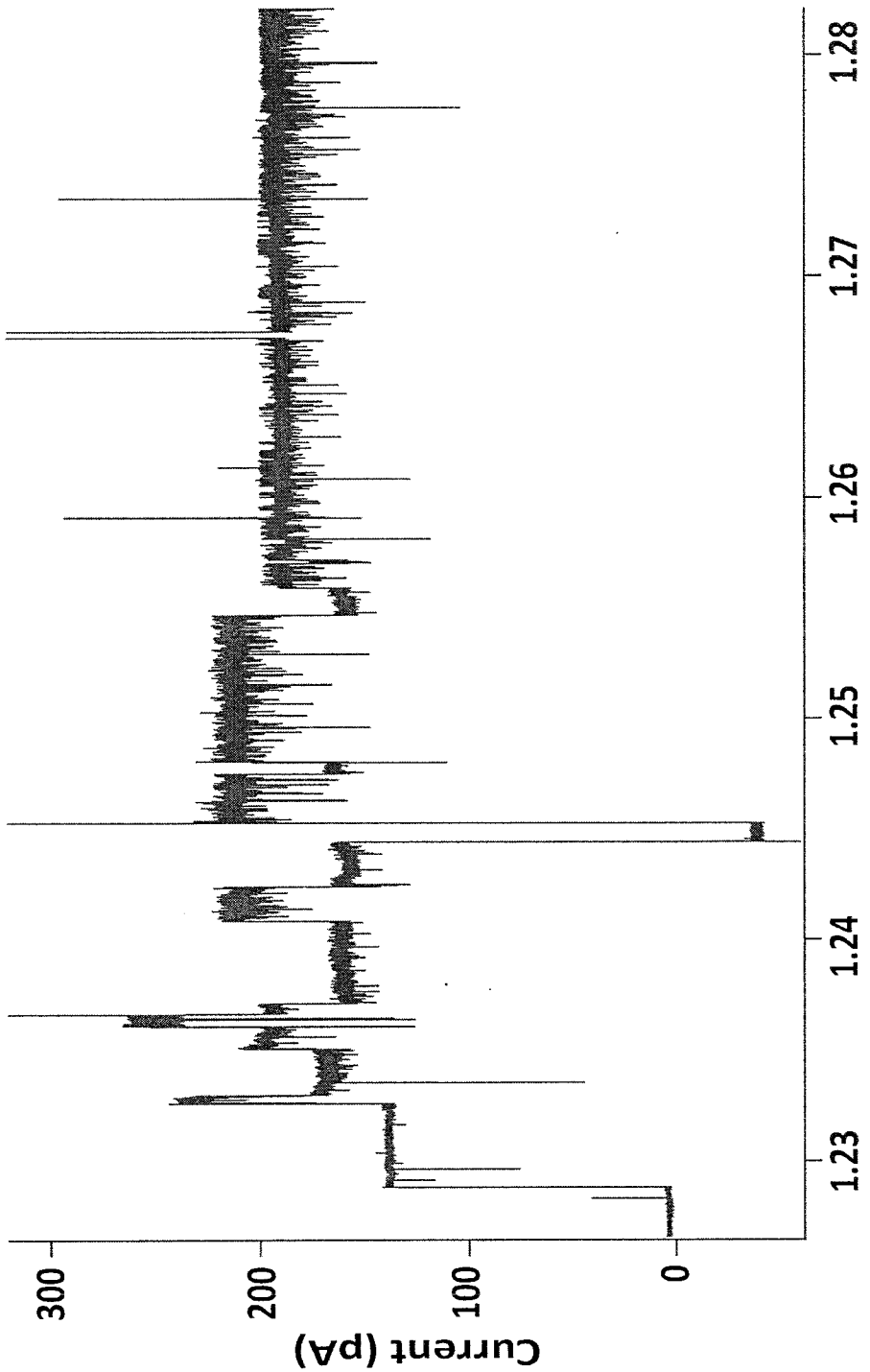
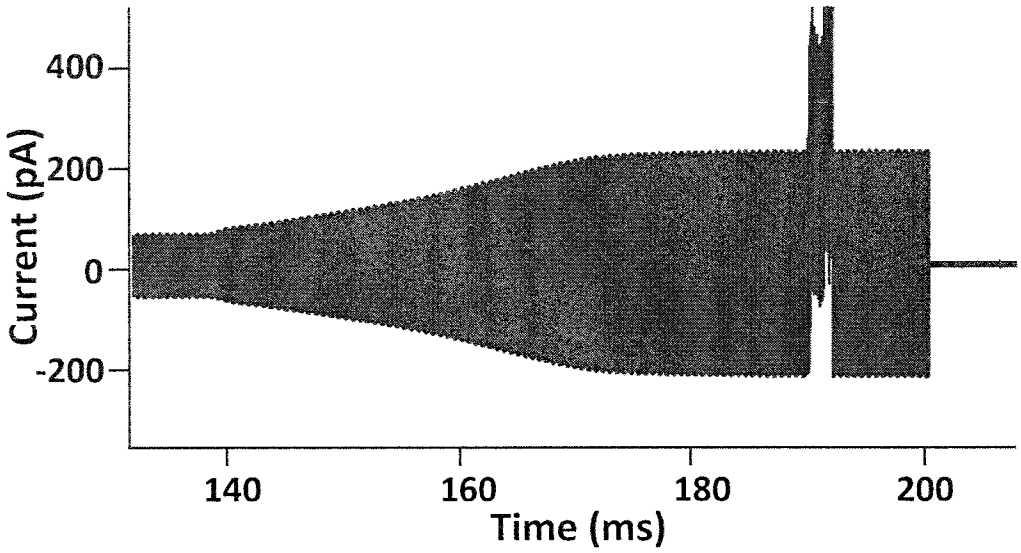
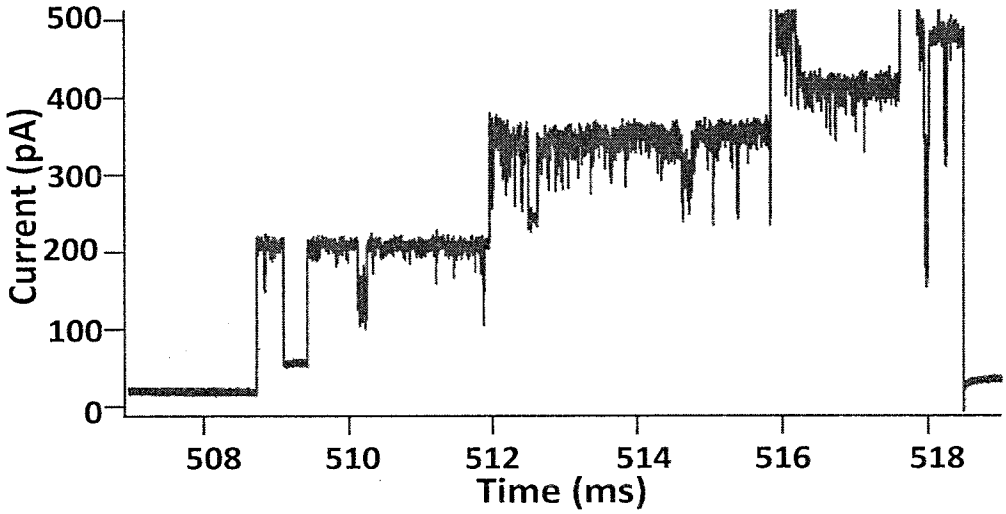


Fig. 6


400
200
0
-200
Current (pA)
140
160
180
200
Time (ms)

A


500
400
300
200
100
0
Current (pA)
508
510
512
514
516
518
Time (ms)

B

Fig. 7