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Lipid nanodiscs useful in solubilising membrane proteins

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(54) **Title:** LIPID NANODISCS USEFUL IN SOLUBILISING MEMBRANE PROTEINS

(57) **Abstract:** Provided herein are composition including lipids and copolymers in the form of a nanodisc assembly. The copolymers include monomer units of methacrylic acid and styrene. Also provided herein is an aqueous solution comprising the subject composition, methods of producing the nanodisc assembly. Further provided are methods of solubilising hydrophobic constituents such as membrane proteins in aqueous solution, including forming nanodisc assemblies of a lipid, the hydrophobic constituent, and the subject copolymer.

LIPID NANODISCS USEFUL IN SOLUBILISING MEMBRANE PROTEINS

Technical field

The present disclosure relates to nanodisc structures of methacrylic acid-co-styrene
5 copolymer and membrane lipids for solubilising membrane proteins.

Background

Structural biology of integral membrane proteins has been developing at a great pace since the resolution revolution of cryo-electron microscopy. Consequently, there has
10 been an increasing interest in reconstituting the membrane proteins in lipid-disc nanoparticles or nanodiscs to help maintain more native-like conditions in solution. The dominating approach is currently solubilisation of the membrane protein with detergents and subsequent re-lipidation of the membrane protein for insertion into a lipid-bilayer encircled by a membrane scaffold protein (MSP). This approach allows the
15 study of the protein in a lipid environment, however, not in the native lipid environment.

Native nanodisc forming polymers are amphipathic polymers capable of dissolving membrane proteins directly from the native lipid bilayer, circumventing the need of detergent. However, this method is still in development, with several shortcomings
20 inherent to the system. Styrene maleic acid (SMA) copolymers, the predominant polymer for this application, are hydrolysed copolymers of styrene and maleic anhydride. These nanodisc forming polymers are sensitive to the presence of divalent cations and have a limited pH range at which they are effective (Scheidelaar, et al. Biophys J (2016), 1 1 1 , 1974-1986). Moreover, proteins embedded in discs formed
25 with SMA have a low affinity to matrices used in affinity-based purification methods, such as Ni-His tag purification or streptavidin affinity columns.

In recent years, research has focused on modifying SMA with functional groups that remedy the issues inherent to SMA (Lee et al. Biochem Soc T 2016, 44, 1011-1018).
30 Furthermore, one specific system for solubilising membrane proteins may not be capable of solubilising all proteins sufficiently well for the proteins to be studied. Accordingly, the provision of new nanostructures will potentially allow for solubilising membrane proteins that cannot be studied using the existing systems. Therefore there is a need for alternative materials and techniques for the formation of different native
35 lipid nanodiscs for the study of membrane proteins.

Summary

Disclosed herein are poly(methacrylic acid-co-styrene) copolymers (MAASTY) capable of solubilising membrane proteins, embedding them in a nanodisc structure comprising the copolymer and membrane lipids.

As shown herein, the MAASTY-based nanodiscs are capable of solubilising a set of membrane proteins to a higher extent than a previously reported poly(acrylic acid-co-styrene) copolymer (AASTY)-based nanodisc (WO 2020/257637 A1). The MAASTY-based nanodiscs also show solubilising properties over a broader range of copolymer compositions than the AASTY-based system, indicating that the MAASTY-based nanodiscs constitute a more robust system than the AASTY-based nanodiscs. In certain cases, the MAASTY-based nanodiscs also favour formation of other quaternary protein structures that are not observed for the AASTY-based nanodiscs.

One aspect of the present disclosure provides for a composition comprising:

a. a lipid
b. a poly(methacrylic acid-co-styrene) copolymer

wherein the lipid and the copolymer are in the form of a nanodisc assembly.

One aspect of the present disclosure provides an aqueous solution comprising a composition of the disclosure.

One aspect of the disclosure provides a method of producing the composition of the disclosure, said method comprising contacting of the lipid of the disclosure and copolymer of the disclosure in a solution.

One aspect of the disclosure provides a method of solubilising a hydrophobic constituent in a solution, the method comprising contacting:

a. a lipid,
b. the hydrophobic constituent, and
c. a poly(methacrylic acid-co-styrene) copolymer.

In certain aspects, the subject compositions increase the aqueous solubility of a hydrophobic constituent, by at least 2-fold, at least 3-fold, at least 4-fold, at least 5-fold,

at least 6-fold, at least 7-fold, at least 8-fold, at least 9-fold, or at least 10-fold compared to the solubility of the hydrophobic constituent in the absence of the subject composition.

5 One aspect of the disclosure provides a use of a poly(methacrylic acid-co-styrene) copolymer for solubilising a protein, such as a membrane protein.

One aspect of the disclosure provides a kit comprising a plurality of poly(methacrylic acid-co-styrene) copolymers, wherein at least two of said poly(methacrylic acid-co-
10 styrene) copolymers differ in their composition of methacrylic acid and styrene.

Description of Drawings

FIG 1. A: Graphic overview of the types of membrane proteins contained in the test set in this study. B: Overview of the preparation of membrane protein-loaded, native
15 nanodiscs as visualized and judged with Fluorescence Size Exclusion Chromatography (FSEC) starting from HEK293 cells overexpressing a membrane protein tagged with Green Fluorescent Protein (GFP). The framed inserts show a tetrameric membrane protein in cells and in native nanodiscs and FSEC traces from its solubilisation in 0.5, 1 and 2% (w/v) of the $MAASTY_{7.5}$-45 copolymer. The figure shows a graphic
20 representation of the protocol.

FIG 2. A: Raw FSEC traces from 8 µL injections of a homotetrameric protein solubilized with 1% (w/v) of the entire MAASTY library. The void volume (^), and the peaks with the oligomeric state expected for the protein (*) are indicated. The figure shows that
25 $MAASTY_{7.5}$-45, $MAASTY_{7.5}$-50, $MAASTY_{7.5}$-55 and $MAASTY_{7.5}$-60 achieved maximum GFP signal of approximately 90000 uV at approximately 11 min, whereas $MAASTY_{7.5}$-40 achieved a GFP signal of approximately 75000 uV and $MAASTY_{7.5}$-65 of 40000 uV at the same time point. B: Raw FSEC traces of the tetrameric membrane protein in the entire AASTY library and $MAASTY_{7.5}$-45 for comparison. The void volume (^), and the
30 peaks with the oligomeric state expected for the protein (*) are indicated. The figure shows that $MAASTY_{7.5}$-45 achieves the highest GFP signal at approximately 11 min of 90 kUV, followed by $AASTY_{11}$-50 at approximately 60 kUV, $AASTY_{11}$-45, $AASTY_{6}$-50, $AASTY_{6}$-55 and $AASTY_{11}$-55 at approximately 30 kUV and $AASTY_{6}$-45 at <20 kUV. C: Raw FSEC traces of 8 µL injections of a heterodimeric membrane protein tagged with
35 GFP and a Red Fluorescent Protein (RFP), respectively. The protein was solubilized in

1% AASTY and MAASTY. The void volume (^) and the peaks corresponding to dimers (*) and monomers (#) are indicated. The presence of dimeric structures was more pronounced in the MAASTY-discs than the in AASTY nanodiscs in both the GFP-labelled and the RFP-labelled study. D: Raw FSEC traces of 8 µL injections of a homodimeric membrane protein solubilized in 1% AASTY and MAASTY tagged with RFP. The void volume (^), dimers (*) and monomers (#) are indicated. The figure shows that the dimeric protein structure is more pronounced in the MAASTY-based nanodisc assembly than in the AASTY-based system.

FIG 3. ^{1}H-NMR (D_2O, 400 MHz): δ [ppm] = 7.5 - 6.5 (phenyl H), 3.0 - 0.3 (aliphatic H). Styrene dyad content is seen in f, with an increased broadening of phenyl signals as the styrenic content increases. The methyl signal from methacrylic acid is seen increasing in proportion with methacrylic acid content. A: $MAASTY_{7.5}$-40; B: $MAASTY_{7.5}$-45; C: $MAASTY_{7.5}$-50; D: $MAASTY_{7.5}$-55; E: $MAASTY_{7.5}$-60; $MAASTY_{7.5}$-65.

FIG 4. Cryo-EM structure of hTRPM4 recorded using MAASTY nanodiscs. A: Example micrograph of hTRPM4 in MAASTY nanodiscs. B: Representative 2D class averages of hTRPM4 in MAASTY nanodiscs. Side (C) and top (D) views of a sharpened cryo-EM density map of hTRPM4 in MAASTY nanodiscs, with subunits (1-4). E: Zoom in on helix S6 to show side chain densities of the cryo-EM map.

Detailed description

Definitions

As used herein, the term “salt” can mean a salt be derived from inorganic or organic bases and from inorganic or organic acids. The term “salt” encompasses pharmaceutically acceptable salts of a compound, which salts are derived from a variety of organic and inorganic counter ions well known in the art and include, by way of example only, sodium, potassium, calcium, magnesium, ammonium, tetraalkylammonium, and the like; and when the molecule contains a basic functionality, salts of organic or inorganic acids, such as hydrochloride, hydrobromide, formate, tartrate, besylate, mesylate, acetate, maleate, oxalate, and the like.

The term “copolymer” is one of the art. It refers to a polymer comprising two or more different monomer units that are polymerized in a process called copolymerization.

Since a copolymer comprises at least two different monomer units, copolymers can be classified based on how the monomer units are arranged to form a polymer chain. Those classifications include “alternating copolymers” (in which the monomers units repeat with a highly regular alternating pattern), “periodic copolymers” (in which the monomers units are arranged with a repeating sequence), “statistical copolymers” (in which the sequence of monomer units follows a statistical rule), “random copolymers” (in which the monomer units are attached in a random order), and “block copolymers” (in which two or more homopolymer subunits are linked).

The term “X comprises in the range of n to m of Y” and similar expressions as used herein refers to that X contains at least n and at the most m of Y. I.e. the term indicates that X does not contain more than m of Y. By way of example, if a compositions is stated to comprise in the range of 5 to 60 mol% styrene, then said composition does not contain more than 60 mol% styrene.

As used herein, the terms “nanodisc assembly” and “nanodisc” refer to at least one lipid bilayer that is stabilized by a synthetic polymer. The synthetic polymer can be a copolymer of styrene and acrylic acid, methacrylic acid, or an acrylic acid derivative, e.g., as disclosed herein. The nanodiscs of the present disclosure are less than one micron in diameter, such as 5-40 nm. The nanodiscs can optionally contain additional lipid components, membrane proteins, and/or proteins that are not membrane proteins. The nanodisc assembly of the disclosure may comprise a membrane protein having a thin lipid annulus around the protein, said annulus having the copolymer of the disclosure coiled around it. In one embodiment, such annulus is considered a lipid bilayer.

Disclosed herein is the use of a subject copolymer, poly(methacrylic acid-co-styrene) (MAASTY) copolymer, to effectively make regularly sized lipid-polymer disc-shaped particles by incubation of the polymer with lipid bilayers, including living cell membranes. In some embodiments, the polymer is made through Reversible Addition Fragmentation chain Transfer polymerization (RAFT), with subsequently modified end groups. In some embodiments, the ratio of functional groups on the subject copolymers are close to equimolar. The subject nanodiscs are formed upon incubation of the subject copolymer with the lipid bilayers, be it from purified membranes, directly from

living cells or organelles. In some cases, the subject nanodiscs formed contain membrane proteins.

The terms “copolymer” and “polymer” may be used interchangeably herein.

5

By “methacrylic acid” is meant the compound having the CAS registry number 79-41-4.

Whenever the term “membrane protein” or “protein” is used, it is within the scope of the present disclosure that the term may also extend to other hydrophobic constituents as
10 described here. While membrane proteins and other proteins may have hydrophilic domains, it is construed that they can be “hydrophobic constituents” provided they have at least one hydrophobic domain.

Molecular weight and molecular mass are used interchangeably herein.

15

Nanodisc assembly of the disclosure

The nanodiscs of the present disclosure are synthetic model membrane systems which assists in the study of membrane proteins. They comprise a lipid bilayer ‘disc’ having its hydrophobic edge screened from the surrounding (aqueous) solution by a ‘belt’ of a
20 amphipathic copolymer.

One embodiment of the present disclosure provides for a composition comprising:

a. a lipid
b. a poly(methacrylic acid-co-styrene) copolymer

25 wherein the lipid and the copolymer are in the form of a nanodisc assembly.
The nanodisc assembly of the disclosure is capable of solubilising hydrophobic constituents, such as membrane proteins. In particular, the composition enables solubilisation of membrane proteins without detergents. It also allows for retaining the native lipids and cofactors.

30

The presently disclosed copolymers have been found to possess solubilising properties over a wide range of copolymer compositions. In one embodiment of the present disclosure, the composition of the disclosure, wherein the copolymer comprises 25 to 80 mol% methacrylic acid. In one embodiment the copolymer comprises 30 to 75 mol%
35 methacrylic acid, such as 35 to 70 mol% methacrylic acid, such as 38 to 67 mol%

methacrylic acid. In one embodiment the copolymer comprises 25 to 30 mol%, such as 30 to 35 mol%, such as 35 to 40 mol%, such as 40 to 45 mol%, such as 45 to 50 mol%, such as 50 to 55 mol%, such as 55 to 60 mol%, such as 60 to 65 mol%, such as 65 to 70 mol%, such as 70 to 75 mol%, such as 75 to 80 mol% methacrylic acid. The methacrylic acid may be present in the copolymer partly or fully in its anionic form and/or as a salt of the anionic form.

In one embodiment of the present disclosure, the copolymer comprises 20 to 75 mol% styrene. In one embodiment the copolymer comprises 25 to 70 mol% styrene, such as 30 to 65 mol% styrene, such as 33 to 62 mol% styrene. In one embodiment the copolymer comprises 20 to 25 mol%, 25 to 30 mol%, such as 30 to 35 mol%, such as 35 to 40 mol%, such as 40 to 45 mol%, such as 45 to 50 mol%, such as 50 to 55 mol%, such as 55 to 60 mol%, such as 60 to 65 mol%, such as 65 to 70 mol%, such as 70 to 75 mol% styrene.

In one embodiment, the mol% of styrene and/or methacrylic acid is measured using ^1H-NMR.

The copolymer of the present disclosure may contain other monomeric units than methacrylic acid and styrene. However, it is construed that a substantial part of the copolymer consists of methacrylic acid and styrene monomeric units. In one embodiment, at least 60 mol% of the copolymer is methacrylic acid and styrene monomers, such as at least 65 mol%, such as at least 70 mol%, such as at least 75 mol%, such as at least 80 mol%, such as at least 85 mol%, such as at least 90 mol%, such as at least 95 mol%, such as at least 97 mol%, such as at least 99 mol%.
In one embodiment the copolymer comprises less than 40 mol% acrylic acid monomers, such as less than 30 mol%, such as less than 20 mol%, such as less than 10 mol%. In one embodiment the copolymer does not comprise acrylic acid monomers.

In one embodiment of the present disclosure, the copolymer is a random copolymer, a statistical copolymer, an alternating copolymer, or a copolymer having a high regularity of alternating units. An alternating copolymer is a copolymer comprising two species of monomeric units distributed in alternating sequence. A random copolymer is one in which the monomer residues are located randomly in the polymer molecule. Statistical copolymers are copolymers in which the sequential distribution of the monomeric units

obeys known statistical laws. In one embodiment, the copolymer is a linear copolymer. In a preferred embodiment of the present disclosure the copolymer is a random copolymer or a statistical copolymer.

5 The copolymer may coil around the lipid bilayer disc one or more times, i.e., forming a belt-like structure around the lipid bilayer disc. The hydrophobic face of the copolymer serves to sequester the hydrocarbon tails of the lipids away from solvent. The resulting disc shaped particle is aqueously soluble and stable. In one embodiment of the present disclosure the copolymer has a molecular mass of less than 20 kDa, such as less than 10 15 kDa, such as less than 14 kDa, such as less than 13 kDa, such as less than 12 kDa, such as less than 10 kDa, such as less than 9 kDa. In one embodiment of the present disclosure the copolymer has a molecular weight of at least 1 kDa, such as at least 2 kDa, such as at least 3 kDa. In one embodiment the copolymer has a molecular weight of 1 to 15 kDa, such as 2 to 15 kDa, such as 3 to 9 kDa. In one embodiment of the 15 disclosure, the copolymer has a molecular weight of 1 to 2 kDa, such as 2 to 3 kDa, such as 3 to 4 kDa, such as 4 to 5 kDa, such as 5 to 6 kDa, such as 6 to 7 kDa, such as 7 to 8 kDa, such as 8 to 9 kDa, such as 9 to 10 kDa, such as 10 to 11 kDa, such as 11 to 12 kDa, such as 12 to 13 kDa, such as 13 to 14 kDa, such as 14 to 15 kDa, such as 15 to 16 kDa, such as 16 to 17 kDa, such as 17 to 18 kDa, such as 18 to 19 kDa, 20 such as 19 to 20 kDa.

In one embodiment, the molecular weight is the weight average molecular weight. In one specific embodiment, the molecular weight is the weight average molecular weight measured by ^{1}H-NMR spectroscopy or by size exclusion chromatography. In one 25 embodiment, the molecular weight is the number average molecular weight. In one specific embodiment, the molecular weight is the number average molecular weight measured by ^{1}H-NMR spectroscopy or size exclusion chromatography.

In one embodiment of the present disclosure the copolymer has 10 to 160 monomeric 30 units. In one embodiment of the present disclosure the copolymer has 15 to 160 monomeric units, such as 15 to 150, such as 20 to 120, such as 30 to 100 monomeric units. In one embodiment, the copolymer has 10 to 15 monomeric units, such as 15 to 20 monomeric units, such as 20 to 25 monomeric units, such as 25 to 30 monomeric units, such as 30 to 35 monomeric units, such as 35 to 40 monomeric units, such as 40 35 to 45 monomeric units, such as 45 to 50 monomeric units, such as 50 to 55 monomeric

units, such as 55 to 60 monomeric units, such as 60 to 65 monomeric units, such as 65 to 70 monomeric units, such as 70 to 75 monomeric units, such as 75 to 80 monomeric units, such as 80 to 85 monomeric units, such as 85 to 90 monomeric units, such as 90 to 95 monomeric units, such as 95 to 100 monomeric units, such as 100 to 105 monomeric units, such as 105 to 110 monomeric units, such as 110 to 115 monomeric units, such as 115 to 120 monomeric units, such as 120 to 125 monomeric units, such as 125 to 130 monomeric units, such as 130 to 135 monomeric units, such as 135 to 140 monomeric units, such as 140 to 145 monomeric units, such as 145 to 150 monomeric units, such as 150 to 155 monomeric units, such as 155 to 160 monomeric units.

In one embodiment, the number of monomeric units is measured by ^{1}H-NMR spectroscopy. The molecular weight calculated through NMR is the number average molecular weight.

The termini of the disclosed copolymer may be conjugated to further chemical moieties, i.e. terminal groups. Such terminal groups may have been introduced as part of the protocol used to produce the copolymer. It is the amphiphilic properties imparted by the methacrylic acid moieties and the styrene moieties of the copolymer that is important for the formation of nanodiscs, specifically the coiling around the lipid bilayer disc and the solubilising of the thereby formed nanodisc assembly in aqueous medium. Therefore, the terminal groups are not essential for the present disclosure; they may be absent or they may be present, and the exact chemical structure of or functional groups presents in said terminal groups are not important in the context of the present disclosure.

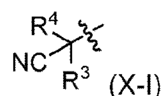
Any convenient group may find use as a terminal group in copolymer of the disclosure. In some embodiments, the terminal groups are each independently a further polymer segment. In certain cases the terminal groups are each independently a terminal group selected from hydrogen, an alkyl or a substituted alkyl. In certain cases, the terminal groups are groups which are produced as a result of any convenient method of polymerisation of the subject co-monomers described herein. In some embodiments the terminal groups comprise a linker that may include a chemoselective functional group. Any convenient methods of derivatising or modifying polymers may be utilised to provide for installation of terminal groups of the subject polymers. In certain cases, the

terminal groups comprise a linked agent, such as a fluorescent dye, or a biomolecule, e.g., biotin, or an antibody.

As used herein, the term “chemoselective functional group” refers to chemoselective
5 reactive groups that selectively react with one another to form a covalent bond. Chemoselective functional groups of interest include, but are not limited to, thiol groups, thiols and maleimide or iodoacetamide, as well as groups that can react with one another via ‘click’ chemistry, e.g., azide and alkyne groups (e.g., cyclooctyne groups). Chemoselective functional groups of interest, include, but are not limited to,
10 thiols, alkyne, a cyclooctyne, an azide, a phosphine, a maleimide, an alkoxyamine, an aldehyde and protected versions thereof, and precursors thereof.

In some embodiments of the copolymer of the disclosure, the terminal groups are selected from, alkyl, substituted alkyl, nitrile, hydroxy, carboxyl, and halogen. In certain
15 embodiments of the copolymer of the disclosure, the terminal groups are selected from alkyl and substituted alkyl. In some cases, the terminal groups are substituted alkyl, and the substituent is selected from one or more of, nitrile, hydroxy, carboxyl, and halogen. In certain cases, the terminal groups are an alkyl group including a nitrile substituent. In certain cases, the terminal groups are an alkyl group including a hydroxy
20 substituent. In certain cases, the terminal groups are an alkyl group including a carboxyl substituent. In certain cases, the terminal groups are an alkyl group including a halogen substituent.

In certain embodiments of the copolymer of the disclosure, the terminal groups is of the
25 formula (X-I):



(X-I)

wherein:

R^3 and R^4 are each independently selected from hydrogen, alkyl, and substituted alkyl. In certain cases of formula (X-I), R^3 and R^4 are both methyl.

30

In some embodiments of the copolymer of the disclosure, the terminal groups are selected from, alkyl, substituted alkyl, nitrile, hydroxy, carboxyl, halogen, thiol, substituted thiol, acyl, and substituted acyl. In certain embodiments of the copolymer of the disclosure, the terminal groups are selected from alkyl and substituted alkyl. In

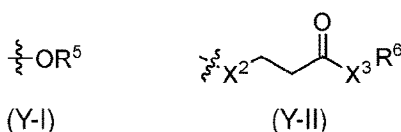
some cases, the terminal groups are substituted alkyl, and the substituent is selected from one or more of alkyl, nitrile, hydroxy, carboxyl, halogen, and thiol. In certain cases, the terminal groups are an alkyl group including a thiol substituent. In certain cases, the terminal groups are an alkyl group including an acyl substituent.

5

In certain embodiments of the copolymer of the disclosure, the terminal groups are selected from, nitrile, hydroxyl, carboxyl, halogen, thiol, substituted thiol, acyl, and substituted acyl. In certain cases, the terminal groups are a nitrile group. In certain cases, the terminal groups are hydroxyl. In certain cases, the terminal groups are
10 carboxyl. In certain cases, the terminal groups are halogen, e.g., Cl, F, I or Br. In certain cases, the terminal groups are acyl or substituted acyl.

In certain cases, the terminal groups are thiol or substituted thiol. In certain cases, the terminal groups are a thiol or substituted thiol that is amenable to conjugation to a
15 fluorescent dye or a biomolecule of interest, e.g., through maleimide chemistry. In certain cases, the biomolecule is a biotin moiety.

In certain embodiments of the copolymer of the disclosure, the terminal groups is of the formula (Y-I) or (Y- II):



20 (Y-I) (Y-II)

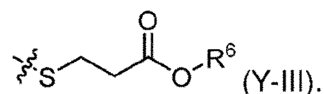
wherein:

R^5 and R^6 are each independently selected from hydrogen, alkyl, and substituted alkyl; and X^2 and X^3 are each independently selected from S and O.

25 In certain embodiments of the formula (Y-I), R^5 is hydrogen. In certain cases, R^5 is alkyl or substituted alkyl. In certain embodiments of formula (Y-II), X^2 and X^3 are both oxygen. In certain cases, X^2 and X^3 are both sulfur. In certain cases, X^2 is S and X^3 is O. In certain cases, X^2 is O and X^3 is S. In certain cases, R^6 is hydrogen. In certain cases, R^6 is alkyl. In certain cases, R^6 is substituted alkyl.

30

In certain embodiments of formula (Y-II), the compound is of the formula (Y-III):



In some embodiments of the copolymer of the disclosure, one or more terminal groups are introduced by reversible addition-fragmentation chain-transfer (RAFT) polymerisation in the preparation of the copolymer of the disclosure using a chain transfer agent. In some cases, the chain transfer agent is 2-(dodecylthiocarbonothioylthio)-2-methylpropionic acid (DDMAT). In one embodiment, one or more end groups of the copolymer of the disclosure is introduced by RAFT chain transfer agents that are cleaved or converted to other chemical groups, including but not limited to thiol, hydroxyl, carboxyl, amine, and alkyl groups. In one embodiment, the end group introduced by the RAFT chain transfer agent is conserved in the copolymer, i.e. it is not further modified or replaced. In one embodiment, the copolymer of formula (I) is prepared via other polymerization methods selected from at least one of anionic polymerization, cationic polymerization, conventional free radical polymerization, or other types of controlled/living free radical polymerization such as atom transfer radical polymerization (ATRP), and nitroxide mediated polymerization (NMP). It will be understood that any convenient means can be utilised to facilitate the preparation of copolymers of the disclosure.

The terminal groups may be the same or may be selected from different moieties defined herein.

In one embodiment of the present disclosure the nanodisc assembly has a diameter of 5 to 40 nm, i.e. they are smaller than one micron in diameter. In one embodiment, the nanodisc assembly has a diameter of 5 to 10 nm, such as 10 to 15 nm, such as 15 to 20 nm, such as 20 to 25 nm, such as 25 to 30 nm, such as 30 to 35 nm, such as 35 to 40 nm. The size of the nanodisc assemblies can be assessed using for example cryo-electron microscopy.

In one embodiment of the present disclosure the composition further comprises a hydrophobic constituent, such as a membrane protein, such as a mammalian membrane protein.

In one embodiment of the disclosure, the nanodisc assembly includes one or more membrane proteins. Membrane proteins that find use in the subject methods include integral membrane proteins, i.e. any protein that crosses, or is embedded or integrated into such membranes, including G-protein coupled receptors (GPCRs), ion channels and transporters. Approximately one third of eukaryotic proteins are associated with membranes in this way. Integral membrane proteins typically have one or more regions which are hydrophobic and lie within the hydrophobic interior of the membrane bilayer, and one or more regions which are hydrophilic and extend out from the membrane. The hydrophilic regions may lie on either or both sides of the membrane. In some cases, the one or more membrane proteins are soluble at a pH<7.0. In some cases, the one or more membrane proteins are soluble in the presence of cations. In some cases, the one or more membrane proteins are adapted for the determination of acidification-induced rhodopsins spectral shifts, activation of ion channels (such as KcsA), or measurement of ATPases. In another embodiment, the one or more membrane proteins are rhodopsins, ion pumps, ATP-binding cassette proteins. In some cases, the one or more membrane proteins are at least one of P-type, F-type, V-type, or ABC ATPases. In one embodiment, the membrane proteins have been modified, such as recombinantly modified, to include a tag, such as a fluorescent tag. Fluorescent tags include GFP and RFP.

Exemplary membrane proteins that can be studied using the nanodisc assemblies of the disclosure also include hTRPM4 (human transient receptor potential melastatin type 4) and hT1R3 (human taste receptor type 1 member 3). The membrane proteins can be conjugated to further moieties facilitating their study, for example fluorescent tags such as green fluorescent protein or red fluorescent protein. The membrane protein to be studied can be a single membrane protein expressed on a cell, or it can be a mixture of membrane proteins expressed on the same cells or on different cells.

In one embodiment of the present disclosure, the lipid is a membrane lipid. The membrane lipid molecules are amphipathic. The most numerous are the phospholipids. When placed in water they assemble spontaneously into bilayers. There are three major classes of membrane lipid molecules: phospholipids, cholesterol, and glycolipids. Thus, in one embodiment the membrane lipid is a phospholipid, a sterol, a glycolipid, or a mixture thereof. The membrane lipid of the disclosure can be of synthetic or natural origin. In certain embodiments of the methods, the lipid is as described herein. In

certain embodiments of the methods, the lipids comprise lipids from purified membranes, living cells or organelles. In some embodiments the lipids are from purified membranes. In some embodiments, the lipids are form living cells. In some cases, the lipids are from organelles. In some cases, the lipid is a single pure component. In some cases, the pure lipid is of synthetic or semi-synthetic origin. In other embodiments, the lipid is a mixture of components. In some cases, the mixture of lipids is of natural origin, e.g., obtained by extraction and purification by means known to those skilled in the art.

The subject compositions may be in the form of an aqueous solution. Thus, one embodiment of the present disclosure provides for an aqueous solution comprising the composition of the disclosure. In some cases, the subject composition is in the form of a stable clear aqueous solution. In some cases, for ease of transportation and handling, once prepared, the compositions may be freeze-dried to form a dry powder which has the benefits of being lower in both volume and weight. In one embodiment of the present disclosure the composition is in the form of an aqueous solution. In a further embodiment of the present disclosure the composition is in freeze-dried form (for example as a powder, resin or flake). Aqueous solutions include aqueous semi-solids such as gels. In one embodiment, the subject composition is in the form of an aqueous solution comprising 0.001 -10% by weight of the compositions disclosed herein (the percentage being determined by the dry weight of composition of the disclosure relative to the total weight of composition and water). In some cases, the subject composition is in the form of an aqueous solution comprising 10-20% by weight of the compositions disclosed herein. In some cases, the subject composition is in the form of an aqueous solution comprising greater than 20% by weight of the compositions disclosed herein.

Methods for producing nanodisc assemblies

The nanodisc assembly of the disclosure can be produced using the protocols disclosed herein. Compositions of the present disclosure may suitably be prepared by mixing a solution of a subject copolymer (e.g., as described herein), with an aqueous emulsion containing lipid, and if necessary adjusting the pH of the resulting mixture such that the polymer/lipid nanodisc assemblies form. The polymer solution may be prepared by dissolving the polymer in water, optionally with stirring and heating. The lipid emulsion may be prepared by mixing dried lipid with water, optionally under stirring and heating (suitably to a temperature above the phase transition temperature of the

lipid component), followed by homogenisation. Suitably the polymer solution and lipid emulsion are mixed by the addition (e.g. the slow addition) of lipid emulsion to the polymer solution, optionally together with heating or cooling. The pH of solutions may be adjusted using acids or bases as appropriate.

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One embodiment of the present disclosure provides for method of producing the composition of the disclosure, wherein said method comprises contacting the lipid of the disclosure and the copolymer of the disclosure in a solution. In one embodiment, the lipid and the copolymer are further contacted with a hydrophobic constituent such
10 as a membrane protein.

In one embodiment of the present disclosure, the solution is an aqueous solution. Other solvents may be present in the aqueous solution, i.e. the solution is a mixture of water and one or more other solvents. The solution may further comprise other solutes
15 such as buffers or salts.

The lipid may be provided as a lipid composition, i.e. a composition comprising the lipid and one or more other components. Similarly, the copolymer can be provided as a copolymer composition comprising the copolymer of the disclosure and one or more
20 other components. In one embodiment of the present disclosure, the lipid is provided in a lipid composition. In one embodiment the copolymer is provided in a copolymer composition. In one embodiment of the present disclosure, the lipid composition and/or the copolymer composition are provided as solutions, such as aqueous solutions.

25 In one embodiment of the present disclosure, wherein the lipid composition and/or the copolymer composition comprises buffer such as HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid) and/or NaOH. In one embodiment of the present disclosure, wherein the concentration of the buffer is 1 to 250 mM, such as 5 to 200 mM, such as 10 to 150 mM, such as 20 to 100 mM. In one embodiment, the
30 lipid composition and/or the copolymer composition comprises a salt such as NaCl. In one embodiment of the disclosure, the concentration of the salt is 10 to 300 mM, such as 20 to 200 mM, such as 50 to 200 mM. In one embodiment of the present disclosure, wherein the lipid composition comprises a protease inhibitor, such as an EDTA-free protease inhibitor.

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In one embodiment of the present disclosure, the lipid composition and/or the copolymer composition have a pH of 6.4 to 10.0, such as 6.9 to 9.5, such as 7.0 to 9.0. In one embodiment of the present disclosure, the lipid composition and/or the copolymer composition have a pH of 6.4 to 7.9, such as 7.9 to 8.4, such as 8.4 to 8.9,
5 such as 8.9 to 9.4, such as 9.4 to 10.0.

In one embodiment of the present disclosure, the lipid composition is a composition comprising a cell, a cell lysate, or a cell membrane such as an isolated cell membrane.

10 It is envisioned that nanodisc assemblies of the present disclosure are capable of solubilising various compounds that are capable of being absorbed into lipid bilayers, solubilised in lipid bilayers, and/or adsorbed onto lipid bilayers. Such are termed hydrophobic constituents herein. The hydrophobic constituent to be studied using the disclosed nanodiscs may originate from a cell, wherein it may naturally be positioned in
15 the lipid bilayer of said cell's membrane. Thus, in one embodiment the hydrophobic constituent, such as the membrane protein, is in a composition comprising a cell, a cell lysate, or a cell membrane such as an isolated cell membrane.

In one embodiment of the present disclosure, wherein the lipid and the hydrophobic
20 constituent such as the membrane protein are in the same composition comprising the cell, the cell lysate, or the cell membrane such as the isolated cell membrane. In one embodiment the lipid originates from the lipid bilayer of the cell's membrane, i.e. the lipid may be a phospholipid, a sterol, a glycolipid, or a mixture thereof, originating from the cell's membrane. While a cell lysate can be employed in formation of the presently
25 disclosed nanodisc assemblies, the cells need not necessarily be actively lysed before the nanodisc assemblies are formed; the copolymer of the disclosure is on its own capable of lysing cell membranes due to its amphiphilic nature.

In one embodiment of the present disclosure, contacting of the lipid and copolymer is
30 carried out under sonication. In one embodiment sonication is carried out for 5 seconds to 5 minutes. The sonication step is optional, though it does provide more efficient contacting/mixing of the components.

In one embodiment of the present disclosure, contacting of the lipid and copolymer
35 comprises incubation. In one embodiment of the present disclosure, incubation is

carried out for at least 0.5 hours, such as at least 1 hour, such as at least 2 hours, such as for 0.5 to 10 hours, such as for 1 to 5 hours, such as for 2 to 4 hours. In one embodiment, incubation is carried out for 0.5 to 1 hour, such as 1 to 2 hours, such as 2 to 3 hours, such as 3 to 4 hours.

In one embodiment of the present disclosure, incubation is carried out at between 1 and 80 °C, such as between 1 and 70 °C, such as between 1 and 60 °C, such as between 1 and 50 °C, for example between 1 and 5 °C, between 5 and 10 °C, between 10 and 15 °C, between 15 and 20 °C, between 20 and 25 °C, between 25 and 30 °C, between 30 and 35 °C, between 35 and 40 °C, between 40 and 45 °C, between 45 and 50 °C, between 50 and 60 °C, between 60 and 70 °C, and/or between 70 and 80 °C. While the nanodisc assemblies comprising the copolymer and the lipid may themselves be stable over a wide range of temperatures, a temperature can be selected based on the thermostability of the membrane protein to be studied.

Larger aggregates of hydrophobic constituents might form during the preparation of the nanodisc assembly. These can be removed by means known to those in the field. In one embodiment, the method of the disclosure further comprises a step of removing large aggregates, such as by centrifugation.

In one embodiment of the present disclosure, the method of the disclosure further comprises the steps of:

a. mixing the copolymer composition and the lipid composition comprising the hydrophobic constituent,
b. optionally sonicating the mixture, and
c. incubating the mixture,

thereby obtaining the composition of the disclosure.

In one embodiment of the present disclosure, the method of the disclosure further comprises the steps of:

a. forming an aqueous solution of:
    i. a poly(methacrylic acid-co-styrene) copolymer comprising 30 to 75 mol% methacrylic acid and 25 to 70 mol% styrene,
    ii. a membrane lipid, and
    iii. a membrane protein,

b. optionally sonicating the mixture for 5 seconds to 5 minutes, and
c. incubating the mixture for 0.5 to 10 hours at 1 to 15 °C,

thereby obtaining the composition of the disclosure.

5 Solutions comprising the nanodisc assembly of the disclosure may be lyophilised to obtain a dry nanodisc assembly composition. Thus, in one embodiment, the method further comprises a step of lyophilising the nanodisc assembly to obtain a dry nanodisc assembly composition

10 Method of solubilizing hydrophobic constituents

In some embodiments, the copolymer is capable of solubilizing hydrophobic constituents into aqueous solvents as disc-shaped nanoparticles. In the case of bilayer lipids, the polymer can create nanodiscs of lipids with membrane proteins embedded. These can be used for the study of membrane proteins through techniques such as 15 cryo-EM and Surface Plasmon Resonance. Another application is the solubilisation of hydrophobic drugs in aqueous solvents. The inventors have surprisingly found that the subject copolymers (e.g., MAASTY copolymers) in the form of a nanodisc assembly are more effective at solubilizing membrane proteins in lipid nanodiscs than poly(acrylic acid-co-styrene) (AASTY) copolymers. In certain cases, the subject copolymers 20 improves the solubility of the membrane proteins at least 2-fold, such as at least 3-fold, at least 4-fold, at least 5-fold, at least 6-fold, at least 7-fold, at least 8-fold, at least 9-fold or at least 10-fold compared to the solubility of the membrane protein in the absence of the subject composition. The subject copolymers provide a high control of monomer sequence, and high regularity of alternating monomer units. As a result, the 25 subject copolymers are capable of consistently solubilizing membrane proteins from lipid bilayers in appreciable quantities.

One embodiment of the present disclosure provides a method of solubilising a hydrophobic constituent in a solution, wherein the method comprises contacting:

30 a. a lipid,
b. the hydrophobic constituent, and
c. a poly(methacrylic acid-co-styrene) copolymer.

One embodiment of the present disclosure provides for use of a poly(methacrylic acid-35 co-styrene) copolymer for solubilising a protein such as a membrane protein.

In one embodiment of the present disclosure, the solubilising comprises forming a nanodisc assembly of the poly(methacrylic acid-co-styrene) copolymer, the protein, and a lipid.

Kits comprising copolymers of the disclosure

The copolymer of the present disclosure can be provided in a kit comprising a plurality of different copolymers or copolymer compositions. A user wishing to find optimal solubilisation conditions for a membrane protein of interest can then test the various different copolymers for their ability to solubilise the specific membrane protein, and thus select the optimal copolymer from the kit. The kit may comprise further copolymers than poly(methacrylic acid-co-styrene) copolymers.

A kit of the disclosure can include a copolymer or a nanodisc (e.g., as described herein); and one or more components selected from the group consisting of a lipid, a buffer, a solvent, a standard, and instructions for use. The one or more components of the kit may be provided in separate containers. The copolymers and nanodiscs of the kits may be provided in a liquid composition, such as any suitable buffer. Alternatively, the copolymers and nanodiscs of the kits may be provided in a dry composition (e.g., may be lyophilized), and the kit may optionally include one or more buffers for reconstituting the dry compound. In one embodiment, the kit may include aliquots of the copolymer or nanodisc provided in separate containers (e.g., separate tubes, bottles, or wells in a multi-well strip or plate).

One embodiment of the present disclosure provides for a kit comprising a plurality of poly(methacrylic acid-co-styrene) copolymers, wherein at least two of said poly(methacrylic acid-co-styrene) copolymers differ in their compositions of methacrylic acid and styrene.

In one embodiment of the present disclosure, the kit further comprises one or more poly(acrylic acid-co-styrene) copolymers, wherein if a plurality of poly(acrylic acid-co-styrene) copolymers are comprised within the kit, at least two of said poly(acrylic acid-co-styrene) copolymers differ in their compositions of acrylic acid and styrene.

In one embodiment of the present disclosure, wherein the one or more poly(methacrylic acid-co-styrene) copolymers are provided as individual aqueous solutions, and wherein if the poly(acrylic acid-co-styrene) copolymers are present, the poly(acrylic acid-co-styrene) copolymers are provided as individual aqueous solutions.

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Examples

Example 1: Formation of lipid nanodiscs containing protein

Materials and methods

Synthesis of MAASTY copolymers

10 Methacrylic acid (MAA) and styrene (STY) were distilled under vacuum. The RAFT agent 2-Methyl-2-[(dodecylsulfanylthiocarbonyl)sulfanyl]propanoic acid (194 mg, 0.531 mmol) and azobisisobutyrunitrile (17.4 mg, 0.106 mmol) were added to six ampoules. Styrene and methacrylic acid were added to the ampoules; A (MAA: 1.7 mL, 1.7 g, 20 mmol, STY: 3.44 mL, 3.13 g, 30 mmol), B (MAA: 1.91 mL, 1.94 g, 22.5 mmol, STY: 3.15 mL, 15 2.85 g, 27.5 mmol), C (MAA: 2.12 mL, 2.15 g, 25 mmol, STY: 2.86 mL, 2.60 g, 25 mmol), D (MAA: 2.33 mL, 2.37 g, 27.5 mmol, STY: 2.58 mL, 2.34 g, 22.5 mmol), E (MAA: 2.55 mL, 2.60 g, 30 mmol, STY: 2.29 mL, 2.10 g, 20 mmol), F (MAA: 2.76 mL, 2.80 g, 32.5 mmol, STY: 2.05 mL, 1.82 g, 17.5 mmol). The ampoules were degassed by four freeze-pump-thaw cycles and sealed under vacuum while frozen. The ampoules were placed in 20 a water bath in a 60 °C oven. This method of heating was chosen as the ampoules were sealed under vacuum, and the monomers will reflux under anisotropic heating. The polymerizations were heated for 17 hours, resulting in yellow solids. The ampoules were shattered, and the yellow solids were dissolved in ethanol. NMR samples for determining conversion were taken at this point. The solutions were precipitated into excess heptane 25 and filtered. The yellow solids were each dissolved in 5 mL ethanol, and 20 eq of H_2O_2 (30% in water) with respect to end-groups were added. These mixtures were heated to 70 °C overnight, resulting in non-colored solutions. These were precipitated into heptane, and the white precipitate recovered by filtration. The polymers were converted to their respective sodium salts, by suspending them in milliQ water, and titrating in 1 M NaOH 30 until the pH was stable at approximately 8, and the bulk of the suspended polymer was dissolved. These solutions were filtered through 0.22 µM PES syringe filters and freeze dried.

Protein expression

hTRPM4 (human transient receptor potential melastatin type 4) fused with an N-terminal StrepTagII and enhanced green fluorescent protein (eGFP), hT1R2 (human taste receptor type 1 member 2) fused with an N-terminal Flag tag and C-terminal eGFP and hT1R3 (human taste receptor type 1 member 3) fused with an N-terminal Flag tag and C-terminal mCherry (red fluorescent protein, RFP), were expressed in suspension HEK293 cells using the baculovirus expression system following the procedure described by Autzen *et al.* 2018. hTRPM4 was expressed in HEK293F cells at 37 °C and enhanced with 10 mM sodium butyrate 24 hours after transduction, while hT1R3 was expressed in HEK293 GnTi$^-$ cells at 33 °C and enhanced with 10 mM sodium butyrate 24 hours after transduction. For both proteins, 1.8 mL from an 850 mL HEK293 culture at a density of 2.8 10^6 cells/mL were transferred to 2 mL centrifuge tubes 48 hours after transduction and pelleted at 3500 *xg* for 10 minutes. Each pellet (~12 mg) was isolated from the media, snap-frozen in liquid nitrogen, and stored at -80 °C until further use.

Fluorescent Size Exclusion Chromatography (FSEC)

Each HEK293 pellet was thawed and resuspended in 250 uL 50 mM Hepes/NaOH adjusted to pH 7.4 and 150 mM NaCl (HBS) supplemented with EDTA-Free SIGMAFAST Protease Inhibitor Cocktail Tablets (Sigma). 50 uL of the cell suspension was mixed with 50 uL polymer solubilized in HBS for a final concentration of 0.5, 1 or 2% (w/v), respectively, and the mix was briefly sonicated with a probe sonicator and incubated on a rolling table for 2-4 hours at 4 °C. Large aggregates were removed from the suspension by ultracentrifugation at 170.000 *xg* for 10 min and the supernatant was filtered with a 0.22 µm spin filter unit before 2 or 8 uL sample was loaded onto a Superose 6 Increase 5/150 GL column (Cytiva) pre-equilibrated with HBS. Separation was performed at a flow rate of 0.15 mL/min and the eluent was detected by a Shimadzu (Shimadzu Europa GmbH) liquid chromatography system equipped with an autosampler (SIL-40), a fluorometer (RF-20A), and a PDA detector (SPD-M40) using excitation wavelengths of 488 nm and 587 nm, and emission wavelengths of 507 nm and 610 nm for detection of eGFP and RFP, respectively, and a recording time of 30 min.

Degree of polymerisation

Degree of polymerisation was assessed by methylating the acid and analysing it with organic SEC, using polystyrene standards. MAASTY polymer (1 equiv.) was suspended in a 1 w% 1-methyl-3-p-tolyltriazene (MTT) solution (1.2 equiv.) in toluene.

The mixture was left to stir overnight. The reaction was finished when all polymer had dissolved in the toluene. Once the reaction was finished, the toluene was washed twice with 1 M HCl and the solvent was removed under vacuum. The degree of methylation was confirmed with NMR. Measurements were performed on a Viscotek Differential
5 Refractometer viscometer with SIL-10AD VP Shimadzu Autosampler and LC-10ADVP Shimadzu Pump using THF as solvent and PLgel 3um MIXED E Column of 300mm x 7,5mm crosslinked porous polystyrene divinylbenzene matrix from Polymer Laboratories. Methylated MAA was dissolved in THF to a concentration of 2.5 mg/mL. Given the synthesis parameters shown, the ranges in terms of DP (degree of
10 polymerization/actual numbers of monomers on average, for each monomer) is shown in Table 2.

Results

Synthesis and chemical characterization of MAASTY

15 We synthesized six MAASTY copolymers with a broad range of compositions, while limiting the molecular weight (M_n) to around 7.5 kDa (Table 1). Following the naming of the AASTY library (Timcenko *et al.* 2022), the MAASTY copolymers are named according to their estimated M_n and the MAA content. Table 2 shows the number of each subunit as assessed by ^{1}H-NMR.

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Table 1. Copolymer characteristics. Abbreviations and symbols used: methacrylic acid, MAA; conversion, conv.; methacrylic acid content, MAA cont.; chemical correlation parameter, λ (λ = 0 is a perfectly random copolymer, λ = −1 is perfectly alternating); number-average molecular weight, M_n.

Polymer	Initial MAA (%)	Conversion (%)	MAA content (%)	λ	M_n, ^{1}H-NMR (kDa)	M_n SEC	Đ
MAASTY$_{7.5}$-40	40	81	44	-0.48	7.4	5.9	1.68
MAASTY$_{7.5}$-45	45	73	48	-0.49	7.0	3.5	1.55
MAASTY$_{7.5}$-50	50	75	51	-0.49	7.1	8.9	1.99
MAASTY$_{7.5}$-55	55	88	55	-0.48	8.2	5.6	1.69
MAASTY$_{7.5}$-60	60	88	59	-0.46	8.1	6.2	1.35
MAASTY$_{7.5}$-65	65	88	63	-0.42	8.0	6.2	1.74

25
Đ: M_w / M_n, where M_w is the weight average molecular weight.

Table 2: degree of polymerisation (number of subunits)

	DP	
MAASTY$_{7.5}$-40	MAA	STY
MAASTY$_{7.5}$-45	22.599	33.898
MAASTY$_{7.5}$-50	25.424	31.073
MAASTY$_{7.5}$-55	28.249	28.249
MAASTY$_{7.5}$-60	31.073	25.424
MAASTY$_{7.5}$-65	33.898	22.599
MAASTY$_{7.5}$-40	36.723	19.774

MAASTY can solubilize mammalian membrane proteins

5 The efficacy of the MAASTY polymers was assessed with FSEC using three different mammalian membrane proteins recombinantly expressed in HEK293 cells: the human transient receptor potential melastatin type 4 (hTRPM4) which functions asa homotetramer, the human sweet receptor (hT1R2/3) which functions as a heterodimer, and the human glucose-sensing receptor (hT1R3/3) which functions as a homodimer. 10 An overview of the protein test set and the FSEC workflow is included in Figure 1A and B, respectively.

hTRPM4 was solubilized with 0.5, 1 and 2% of MAASTY$_{7.5}$-45 (Figure 1B insert) and the entire MAASTY library at a total of 1% (w/v) of all six MAASTY copolymers (Figure 2A). 15 In all cases, hTRPM4 can be extracted in MAASTY in native nanodiscs. In comparison to the AASTY library, MAASTY is more efficient for extracting hTRPM4 in native nanodiscs when evaluating the nanodisc peak (Figure 2B).

The taste receptors hT1R2 and hT1R3 were also solubilized in AASTY$_{11}$-45 and MAASTY$_{7.5}$-45 (Figure 2C and D). The heterodimer composed by hT1R2/hT1R3 is 20 proposed to be the functional sweet receptor in the human tongue, while the homodimeric hT1R3/hT1R3 is proposed to act as a glucose-sensing receptor in the pancreas. In either case, the monomeric form is not expected to be functional. Solubilization of the heterodimer hT1R2/hT1R3 with MAASTY$_{7.5}$-45 produces a dimeric peak (assumed so from comparison with the detergent-solubilized sample), while 25 AASTY$_{11}$-45 also produces a monomeric peak (Figure 2C). Likewise, AASTY$_{11}$-45 favours a monomeric hT1R3 over a dimeric hT1R3 in contrast to MAASTY$_{7.5}$-45 (Figure 2D).

Conclusion

These results show that the MAASTY copolymers are capable of solubilising membrane proteins. The MAASTY polymer was found to solubilise membrane proteins to a higher extent than AASTY. Furthermore, the MAASTY polymer was found to facilitate formation of other quaternary structures (i.e. dimeric structures) which the AASTY copolymer did not.

Example 2: Purification of hTRPM4 in MAASTY lipid nanodiscs

Materials and methods

hTRPM4 in MAASTY nanodiscs were prepared as described Smith *et al.* (Chem 2020, vol 6, issue 10, pages 2782-2795).

Cryo-EM sample preparation and data collection of hTRPM4 in MAASTY lipid nanodiscs

Single particle cryo-electron microscopy (cryo-EM) data of hTRPM4 in MAASTY nanodiscs was collected as described in Autzen *et al.* Science 2018 with the following modifications. Images were recorded by Falcon 4i Electron Detector at a pixel size of 0.749 Å at a defocus range of 0.8-2.5 μm under focus.

Cryo-EM Image processing

Cryo-EM image processing of hTRPM4 in MAASTY nanodiscs was carried out as described in Autzen et al. Science 2018 with few modifications. 5,433 images were collected from which a total of 780,000 particles were picked. These particles were subjected to two rounds of 2D classification and a single round of 3D classification. The best 3D class was further refined in 3D yielding a structure with a global estimated resolution of 3.8 Å (gold standard).

Results

Figure 4 shows the results from the cryo-EM study. A: Example micrograph of hTRPM4 in MAASTY nanodiscs. B: Representative 2D class averages of hTRPM4 in MAASTY nanodiscs. Side (C) and top (D) views of a sharpened cryo-EM density map of hTRPM4 in MAASTY nanodiscs, with subunits (1-4). E: Zoom in on helix S6 to show side chain densities of the cryo-EM map.

Conclusion

A cryo-EM structure was successfully obtained for hTRPM4 in the MAASTY nanodisc assembly.

5 **References**

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Claims

1. A composition comprising:
   a. a lipid
   b. a poly(methacrylic acid-co-styrene) copolymer

   wherein the lipid and the copolymer are in the form of a nanodisc assembly.

2. The composition according to claim 1, wherein the copolymer comprises 25 to 80 mol% methacrylic acid.

3. The composition according to claim 2, wherein the copolymer comprises 30 to 75 mol% methacrylic acid, such as 35 to 70 mol% methacrylic acid, such as 38 to 67 mol% methacrylic acid.

4. The composition according to claim 1, wherein the copolymer comprises 20 to 75 mol% styrene.

5. The composition according to claim 2, wherein the copolymer comprises 25 to 70 mol% styrene, such as 30 to 65 mol% styrene, such as 33 to 62 mol% styrene.

6. The composition according to any one of the preceding claims, wherein the copolymer is a random copolymer, statistical copolymer, alternating copolymer, or a copolymer having a high regularity of alternating units.

7. The composition according to claim 6, wherein the copolymer is a random copolymer or a statistical copolymer.

8. The composition according to any one of the preceding claims, wherein the copolymer has a molecular mass of less than 20 kDa, such as less than 15 kDa, such as less than 14 kDa, such as less than 13 kDa, such as less than 12 kDa, such as less than 10 kDa, such as less than 9 kDa.

9. The composition according to any one of claims 1 to 6, wherein the copolymer has a molecular weight of at least 1 kDa, such as at least 2 kDa, such as at least 3 kDa.

10. The composition according to any one of claims 1 to 6, wherein the copolymer has a molecular weight of 1 to 15 kDa, such as 2 to 15 kDa, such as 3 to 9 kDa.

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11. The composition according to any one of claims 8 to 10, wherein the molecular weight is the number average molecular weight.

12. The composition according to any one of claims 8 to 11, wherein the molecular weight is the number average molecular weight measured by ^{1}H-NMR.

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13. The composition according to any one of claims 8 to 11, wherein the molecular weight is the number average molecular weight measured by size exclusion chromatography.

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14. The composition according to any one of claims 1-7, wherein the copolymer has 10 to 160 monomeric units.

15. The composition according to claim 14, wherein the copolymer has 15 to 160 monomeric units, such as 15 to 150, such as 20 to 120, such as 30 to 100

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monomeric units.

16. The composition according to any one of claims 14 and 15, wherein the number of monomeric units is measured by ^{1}H-NMR.

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17. The composition according to any one of the preceding claims, wherein the nanodisc assembly has a diameter of 5 to 40 nm.

18. The composition according to claim 17, wherein the diameter is measured using cryo-electron microscopy.

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19. The composition according to any one of the preceding claims, wherein the composition further comprises a hydrophobic constituent, such as a membrane protein, such as a mammalian membrane protein.

20. The composition according to any one of the preceding claims, wherein the lipid is a membrane lipid.

21. The composition according to claim 20, wherein the membrane lipid comprises a phospholipid, a sterol, a glycolipid, or a mixture thereof.

22. The composition according to any one of the preceding claims, wherein the copolymer comprises less than 10 mol% acrylic acid monomers.

23. The composition according to any one of the preceding claims, wherein the copolymer does not comprise acrylic acid monomers.

24. An aqueous solution comprising a composition according to any one of the preceding claims.

25. A method of producing the composition according to any one of claims 1 to 23, said method comprising contacting of the lipid and copolymer in a solution.

26. The method according to claim 25, wherein the lipid and the copolymer are further contacted with a hydrophobic constituent such as a membrane protein.

27. The method according to any one of claims 25 to 26, wherein the solution is an aqueous solution.

28. The method according to any one of claims 25 to 27, wherein the lipid is provided in a lipid composition, and/or where the copolymer is provided in a copolymer composition.

29. The method according to claim 28, wherein the lipid composition and/or the copolymer composition are provided as solutions, such as aqueous solutions.

30. The method according to claim 28, wherein the lipid composition and/or the copolymer composition comprises buffer such as HEPES and/or NaOH.

31. The method according to claim 30, wherein the concentration of the buffer is 1 to 250 mM, such as 5 to 200 mM, such as 10 to 150 mM, such as 20 to 100 mM.

5 32. The method according to claim 28, wherein the lipid composition and/or the copolymer composition comprises a salt such as NaCl.

33. The method according to claim 32, wherein the concentration of the salt is 10 to 300 mM, such as 50 to 200 mM.

10

34. The method according to claim 28, wherein the lipid composition comprises a protease inhibitor, such as an EDTA-free protease inhibitor.

35. The method according to any one of claims 25 to 34, wherein the lipid
15 composition and/or the copolymer composition have a pH of 6.4 to 10.0, such as 6.9 to 9.5, such as 7.0 to 9.0.

36. The method according to any one of claims 25 to 35, wherein the lipid composition is a composition comprising a cell, a cell lysate, or a cell
20 membrane such as an isolated cell membrane.

37. The method according to any one of claims 25 to 36, wherein the lipid is a membrane lipid such as a phospholipid, a sterol, a glycolipid, or a mixture thereof.

25

38. The method according to any one of claims 25 to 37, wherein the hydrophobic constituent, such as the membrane protein, is in a composition comprising a cell, a cell lysate, or a cell membrane such as an isolated cell membrane.

30 39. The method according to any one of claims 25 to 38, wherein the lipid and the hydrophobic constituent such as the membrane protein are in the same composition comprising the cell, the cell lysate, or the cell membrane such as the isolated cell membrane.

40. The method according to any one of claims 25 to 39, wherein said contacting comprises sonication.

41. The method according to claim 40, wherein said sonication is carried out for 5 seconds to 5 minutes.

42. The method according to any one of claims 25 to 41, wherein said contacting comprises incubation.

43. The method according to claim 42, wherein said incubation is carried out for at least 0.5, such as at least 1 hour, such as at least 2 hours, such as for 0.5 to 10 hours, such as for 1 to 5 hours, such as for 2 to 4 hours.

44. The method according to any one of claims 42 and 45, wherein said incubation is carried out at between 1 and 80 °C, such as between 1 and 70 °C, such as between 1 and 60 °C, such as between 1 and 50 °C, for example between 1 and 5 °C, between 5 and 10 °C, between 10 and 15 °C, between 15 and 20 °C, between 20 and 25 °C, between 25 and 30 °C, between 30 and 35 °C, between 35 and 40 °C, between 40 and 45 °C, between 45 and 50 °C, between 50 and 60 °C, between 60 and 70 °C, and/or between 70 and 80 °C.

45. The method according to any one of claims 25 to 44, said method further comprising a step of removing large aggregates, such as by centrifugation.

46. The method according to any one of claims 26 to 45 comprising the steps of:
    a. mixing the copolymer composition and the lipid composition comprising the hydrophobic constituent,
    b. optionally sonicating the mixture, and
    c. incubating the mixture,

thereby obtaining the composition according to any one of claims 1 to 23.

47. The method according to any one of claims 26 to 46 comprising the steps of:
    a. forming an aqueous solution of:
        i. a poly(methacrylic acid-co-styrene) copolymer comprising 30 to 75 mol% methacrylic acid and 25 to 70 mol% styrene,

ii. a membrane lipid, and
iii. a membrane protein,

b. optionally sonicating the mixture for 5 seconds to 5 minutes, and
c. incubating the mixture for 0.5 to 10 hours at 1 to 15 °C,

5 thereby obtaining the composition according to claim 19.

48. The method according to any one of claims 26 to 47, wherein the method further comprises a step of lyophilising the nanodisc assembly to obtain a dry nanodisc assembly composition.

10

49. A method of solubilizing a hydrophobic constituent in a solution, the method comprising contacting:
   a. a lipid,
   b. the hydrophobic constituent, and
15 c. a poly(methacrylic acid-co-styrene) copolymer.

50. The method according to claim 49, wherein the hydrophobic constituent is a membrane protein.

20 51. Use of a poly(methacrylic acid-co-styrene) copolymer for solubilising a protein.

52. The use according to claim 51, wherein the solubilising comprises forming a nanodisc assembly of the poly(methacrylic acid-co-styrene) copolymer, the protein, and a lipid.

25

53. The use according to any one of claims 51 and 52, wherein the protein is a membrane protein.

54. A kit comprising a plurality of poly(methacrylic acid-co-styrene) copolymers,
30 wherein at least two of said poly(methacrylic acid-co-styrene) copolymers differ in their compositions of methacrylic acid and styrene.

55. The kit according to claim 54, wherein the kit further comprises one or more poly(acrylic acid-co-styrene) copolymers, wherein if a plurality of poly(acrylic
35 acid-co-styrene) copolymers are comprised within the kit, at least two of said

poly(acrylic acid-co-styrene) copolymers differ in their compositions of acrylic acid and styrene.

56. The kit according to any one of claims 54 and 55, wherein the one or more poly(methacrylic acid-co-styrene) copolymers are provided as individual aqueous solutions, and wherein if the poly(acrylic acid-co-styrene) copolymers are present, the poly(acrylic acid-co-styrene) copolymers are provided as individual aqueous solutions.

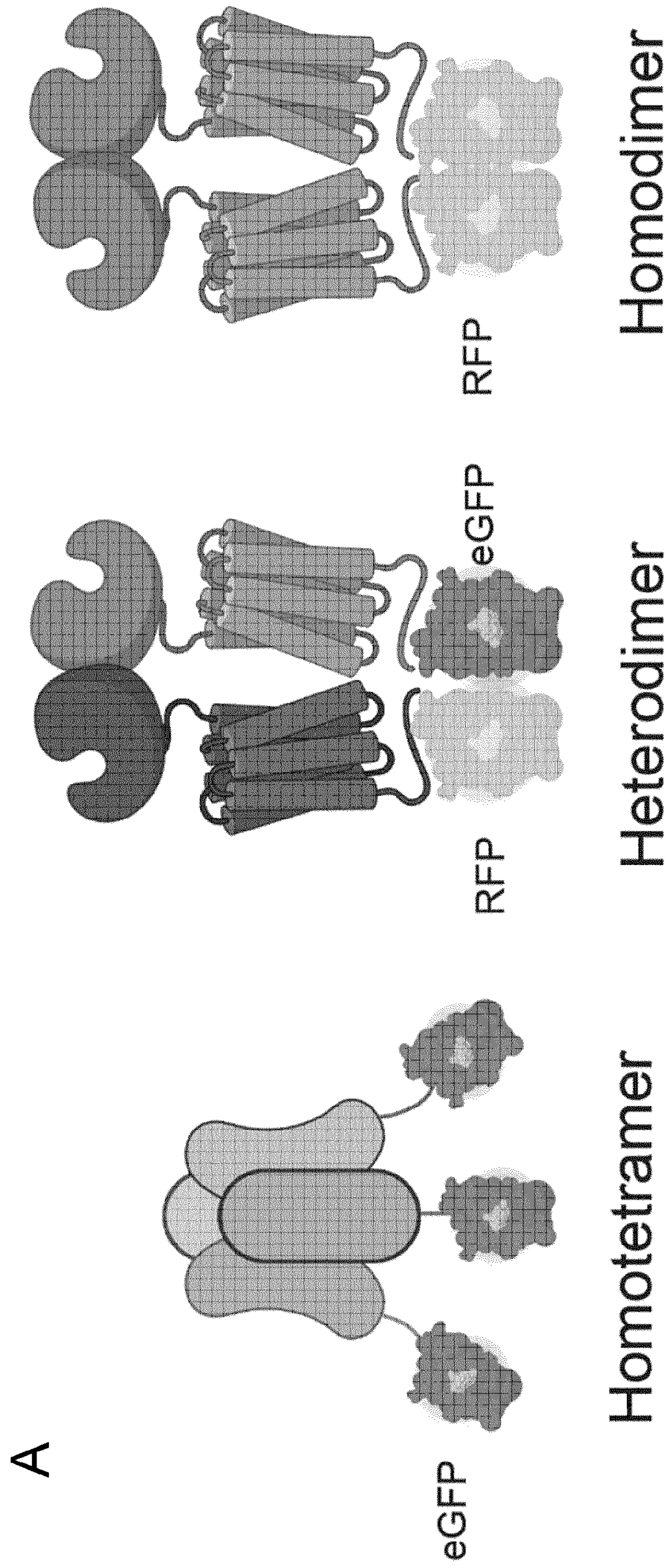


FIG 1

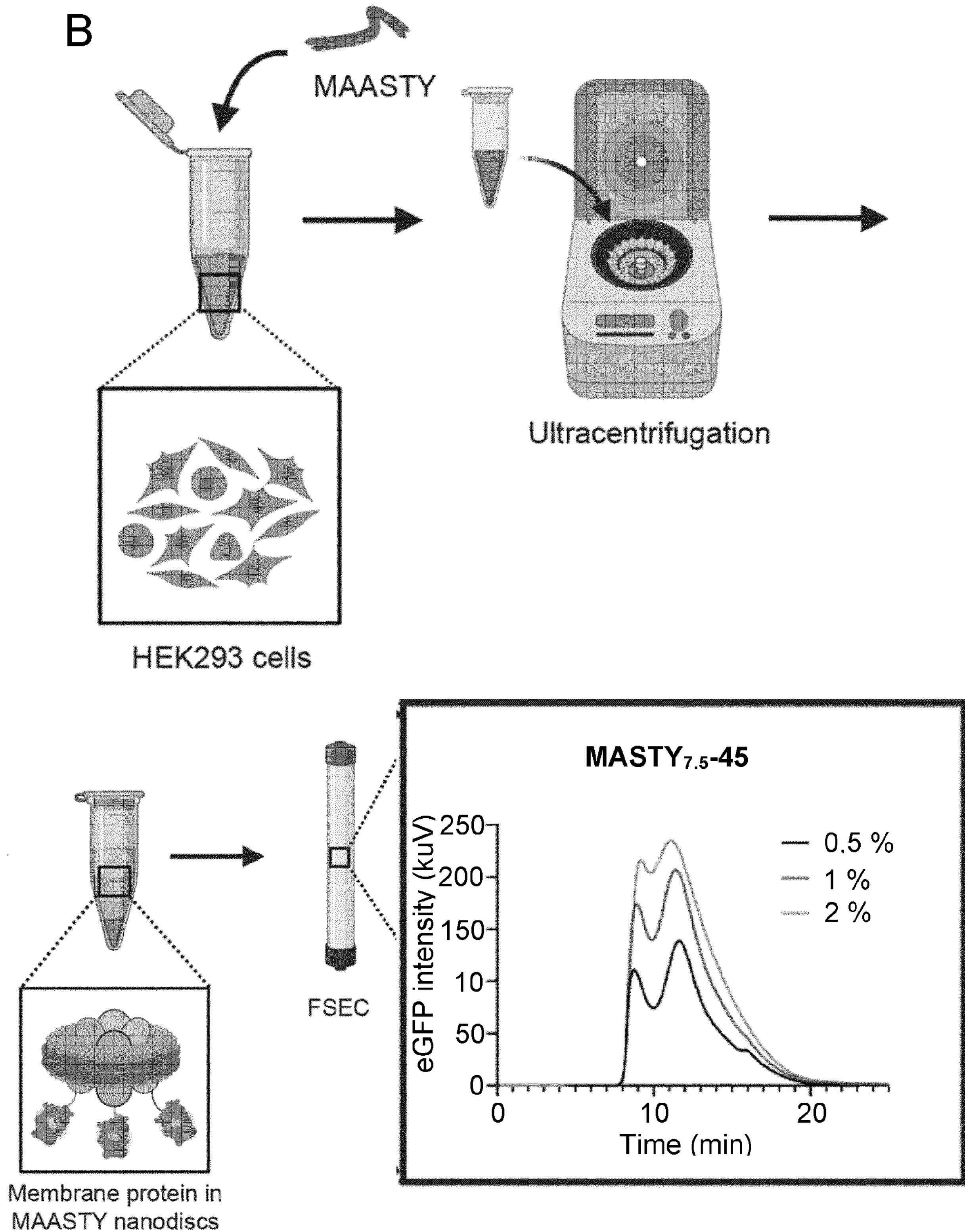


FIG 1 (cont.)

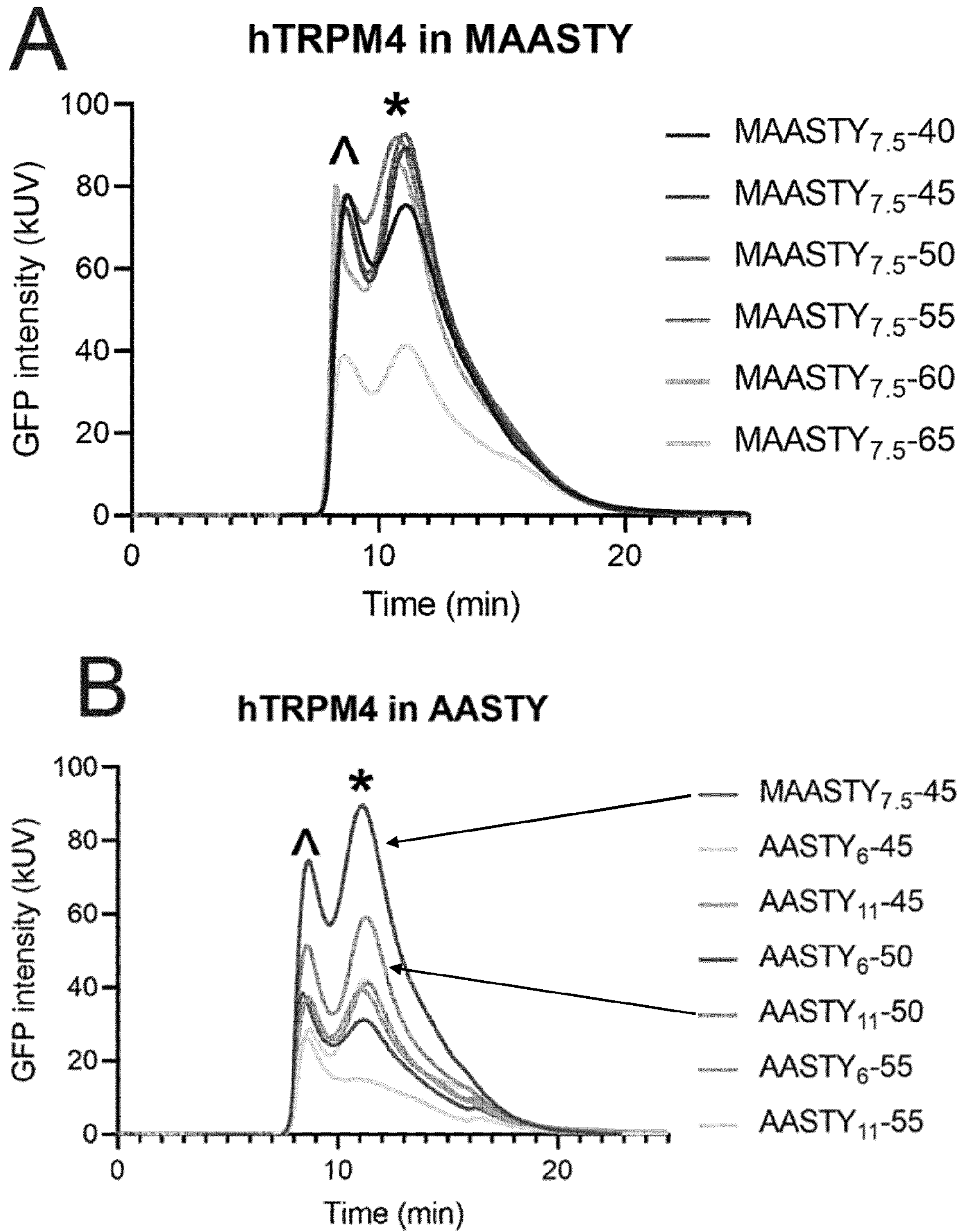
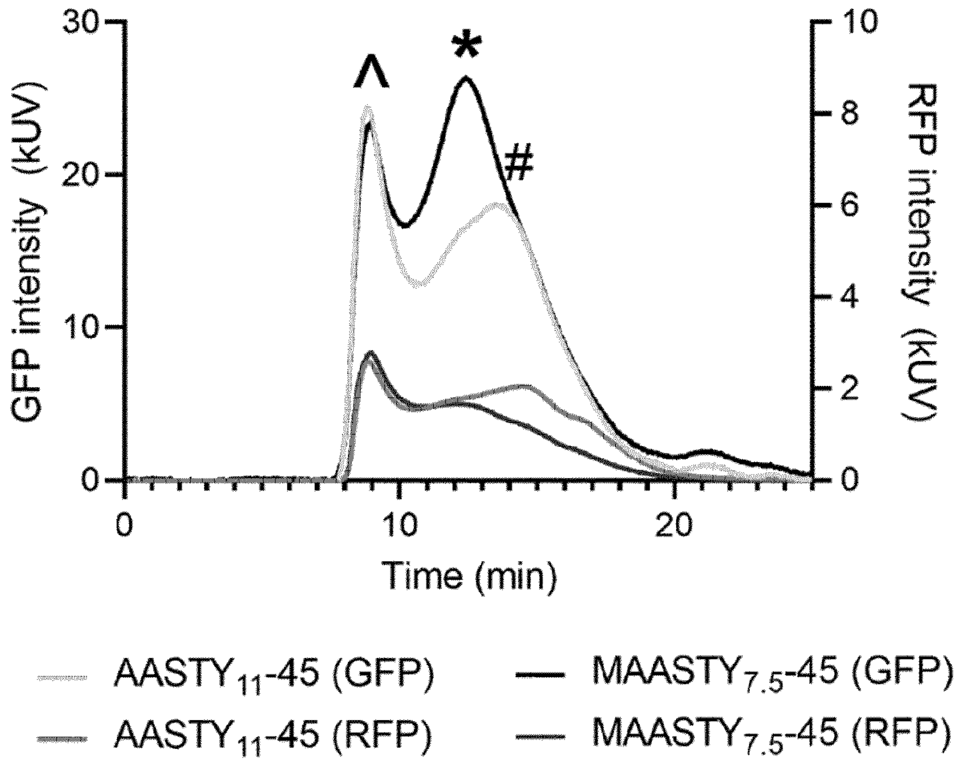


FIG 2



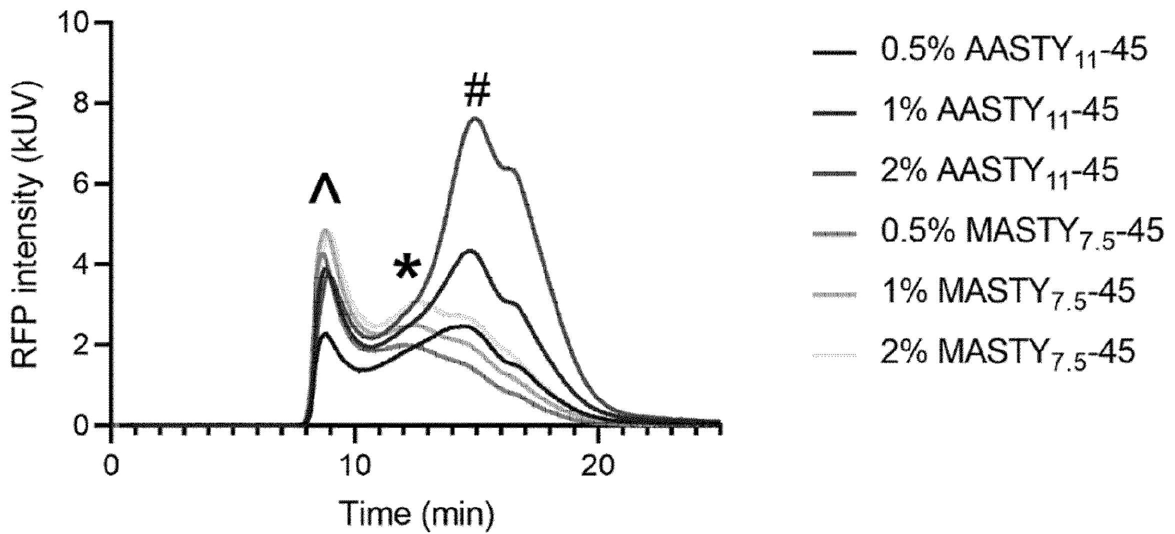
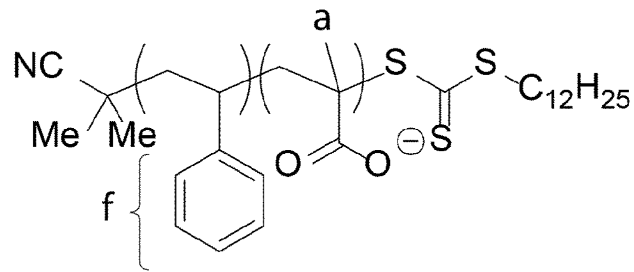


FIG 2 (cont.)



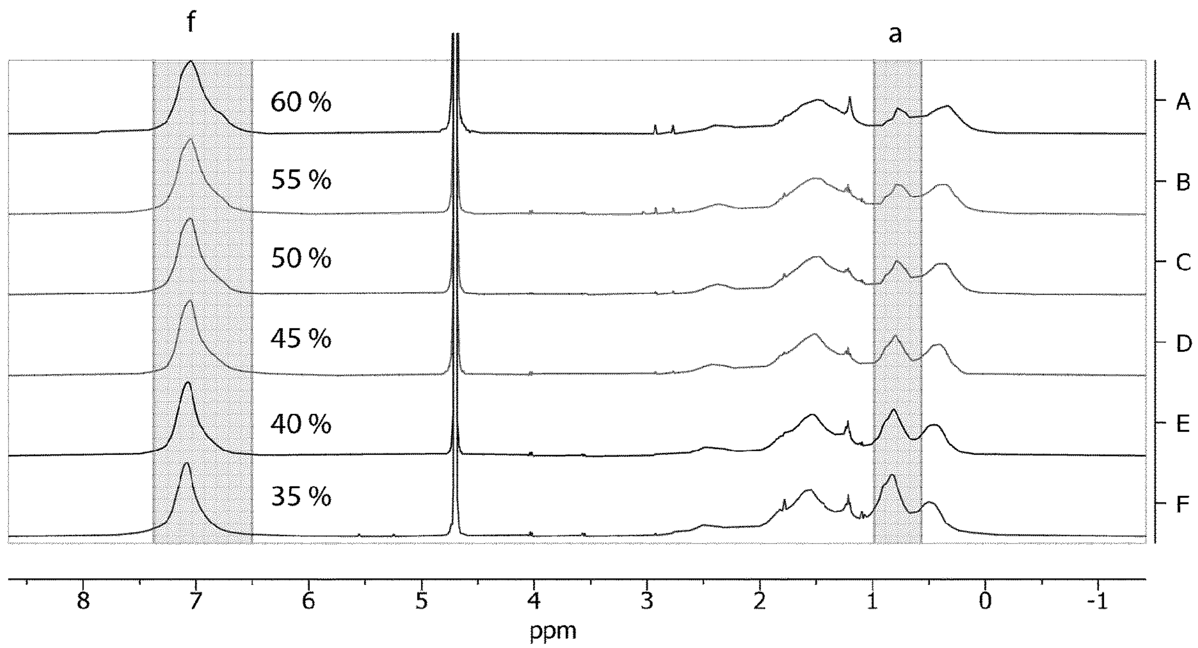
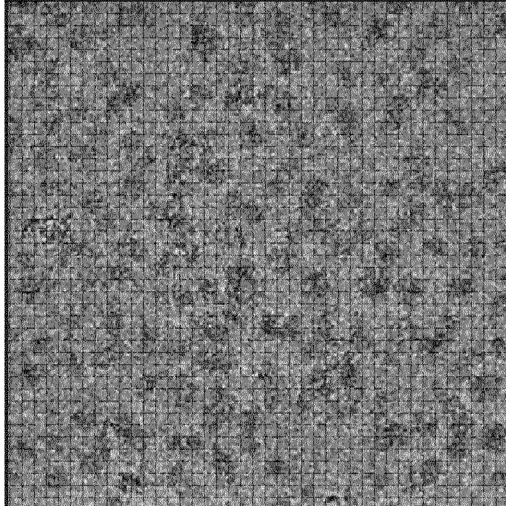


FIG 3

A



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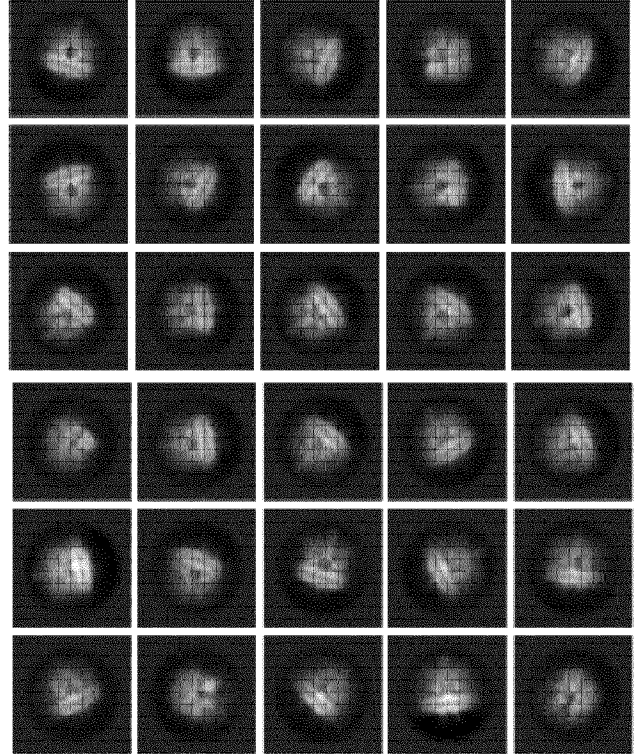
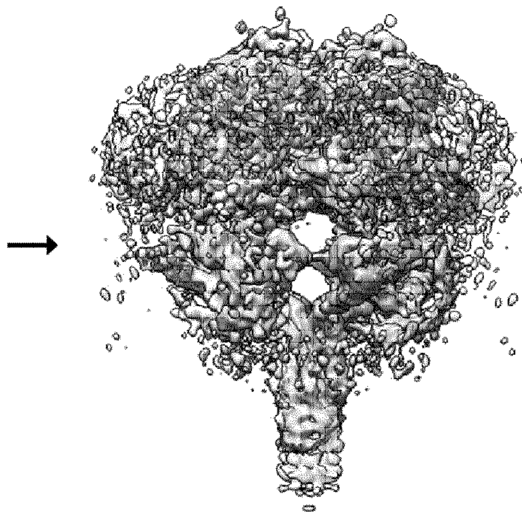
Pixel size (Å): 0.749
Defocus range (μm): 0.8-2.5
Micrographs: 5,433

B



C



D

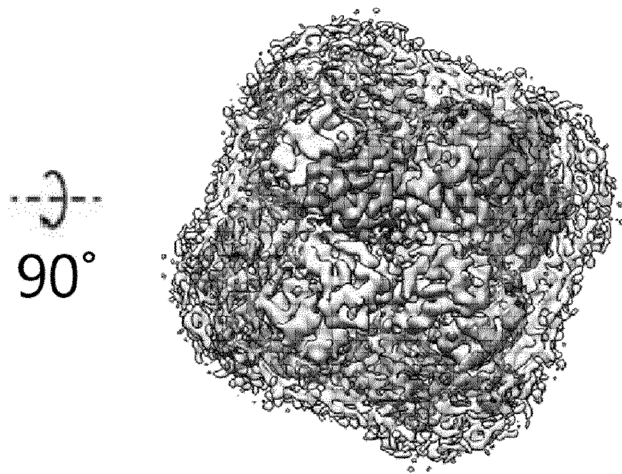


FIG 4

E

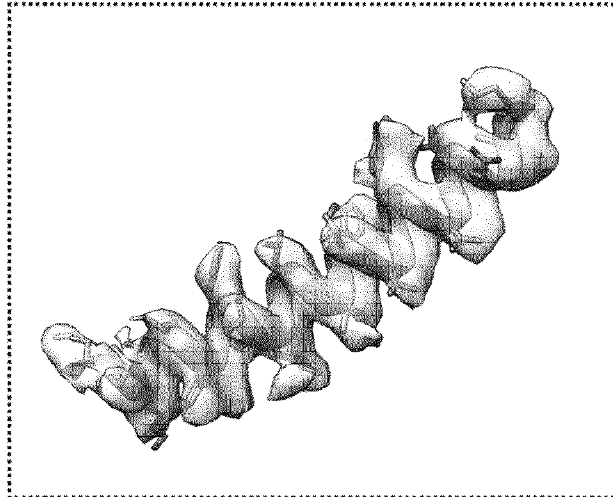


FIG 4 (cont.)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2024/051150

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K9/51 C08F212/08 C08F220/06 C08L25/08 C08L33/02
G01N33/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K G01N C09J C08F C08L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2020/257637 A1 (APPEL ERIC A [US]; SMITH ANTON [US] ET AL.) 24 December 2020 (2020-12-24) cited in the application paragraphs [0003], [0004], [0035] - [0036], [0124] - [0130]; claims 1,4,19,23,24,25,47-48, 49-50; table 1 -----	1-56

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[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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Date of the actual completion of the international search	Date of mailing of the international search report
4 March 2024	21/03/2024

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Authorized officer
Jegou, Gwénaëlle

1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Polymer Source Ch: "Sample Name: Random Copolymer Poly(styrene-co- methacrylic acid) Sample #: P7413-SMAAran Structure: DSC thermogram for the sample: Temperature (°C)", , 5 February 2019 (2019-02-05), XP093053089, Retrieved from the Internet: URL:https://www.polymersource.ca/index.php?route=product/category&path=2_20_127_2229_1090&subtract=1&categorystart=A-3.4.3.24&serachproduct= [retrieved on 2023-06-09] page 1 - page 1	2-23
Y	Polymer Source Ch: "Sample Name: Random Copolymer Poly(styrene-co- methacrylic acid) Sample #: P7416-SMAAran Structure: solvent trace DSC thermogram for the sample: Temperature (°C)", , 5 February 2019 (2019-02-05), XP093053084, Retrieved from the Internet: URL:https://www.polymersource.ca/index.php?route=product/category&path=2_20_127_2229_1090&subtract=1&categorystart=A-3.4.3.24&serachproduct= [retrieved on 2023-06-09] page 1 - page 1	2-23
Y	FORSTER S ET AL: "Amphiphilic block copolymers in structure-controlled nanomaterial hybrids", ADVANCED MATERIALS, VCH PUBLISHERS, DE, vol. 10, no. 3, 11 February 1998 (1998-02-11), pages 195-217, XP002121144, ISSN: 0935-9648, DOI: 10.1002/(SICI)1521-4095(199802)10:3<195::AID-ADMA195>3.0.CO;2-V figure 1	1-56
Y	YESSINE M-A ET AL: "Characterization of the membrane-destabilizing properties of different pH-sensitive methacrylic acid copolymers", BIOCHIMICA ET BIOPHYSICA ACTA, ELSEVIER, AMSTERDAM, NL, vol. 1613, no. 1-2, 27 June 2003 (2003-06-27), pages 28-38, XP004433589, ISSN: 0005-2736, DOI: 10.1016/S0005-2736(03)00137-8 paragraph [04.2]	1-56

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1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KOPF ADRIAN H. ET AL: "Synthesis and Evaluation of a Library of Alternating Amphipathic Copolymers to Solubilize and Study Membrane Proteins", BIOMACROMOLECULES , vol. 23, no. 3 7 January 2022 (2022-01-07), pages 743-759, XP093052972, US ISSN: 1525-7797, DOI: 10.1021/acs.biomac.1c01166 Retrieved from the Internet: URL:https://pubs.acs.org/doi/pdf/10.1021/acs.biomac.1c01166 scheme 1 abstract; figures 1-2; table 2 -----	1-56
A	BROWN CHANELLE J. ET AL: "Structural biology of endogenous membrane protein assemblies in native nanodiscs", CURRENT OPINION IN STRUCTURAL BIOLOGY, vol. 69, 1 August 2021 (2021-08-01), pages 70-77, XP093052973, GB ISSN: 0959-440X, DOI: 10.1016/j.sbi.2021.03.008 figure 1; tables 1-2 -----	1-56
A	SMITH ANTON A.A. ET AL: "Lipid Nanodiscs via Ordered Copolymers", CHEM, vol. 6, no. 10, 1 October 2020 (2020-10-01), pages 2782-2795, XP093052980, US ISSN: 2451-9294, DOI: 10.1016/j.chempr.2020.08.004 figures 1-2; table 1 -----	1-56

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2024/051150

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2020257637 A1	24-12-2020	AU 2020298299 A1	24-02-2022
		CA 3143709 A1	24-12-2020
		EP 3986941 A1	27-04-2022
		US 2022251261 A1	11-08-2022
		WO 2020257637 A1	24-12-2020