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(71) Applicant: **THE UNIVERSITY OF BRISTOL** [GB/GB]; Senate House, Tyndall Avenue, Bristol BS8 1TH (GB).

(72) Inventors: **PERRIMAN, Adam, Willis**; c/o The University of Bristol, Research and Enterprise Development, One Cathedral Square, Bristol BS1 5DD (GB). **DELLER, Robert, Christopher**; c/o The University of Bristol, Research and Enterprise Development, One Cathedral Square, Bristol BS1 5DD (GB). **XIAO, Wenjin**; c/o The University of Bristol, Research and Enterprise Development, One Cathedral Square, Bristol BS1 5DD (GB). **GREEN, Thomas, Iain, Phillip**; c/o The University of Bristol, Research and Enterprise Development, One Cathedral Square, Bristol BS1 5DD (GB). **CARTER, Benjamin, Michael**; c/o The University of Bristol, Research and Enterprise Development, One Cathedral Square, Bristol BS1 5DD (GB). **DAY, Graham, John**; c/o The University of Bristol, Research and Enterprise Development, One Cathedral Square, Bristol BS1 5DD (GB). **DELINT, Rosalia, Cuahtecontzi**; c/o The University of Bristol, Research and Enterprise Development, One Cathedral Square, Bristol BS1 5DD (GB).

(74) Agent: **TURNER, Rhiannon** et al.; Greaves Brewster LLP, Copa House, Station Road, Cheddar Somerset BS27 3AH (GB).

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(54) Title: PROTEIN DELIVERY TO MEMBRANES

(57) Abstract: There is provided a phospholipid composition which is a bilayer or micelle comprising at least one embedded protein-polymer surfactant conjugate comprising an anchor protein, wherein the anchor protein is a cationised protein or an anionised protein, the composition characterised in that the anchor protein is: a) an active enzyme; or b) is a protein which does not comprise a $-CH_2C(O)NCH_3(CH_2)_3N(CH_3)_2H^+$ linker covalently bonded to an amino acid side chain.

PROTEIN DELIVERY TO MEMBRANES

TECHNICAL FIELD

The invention relates to novel methods of positioning a protein such as an active enzyme into a phospholipid structure such as a cell membrane. It also relates to structures arising from the method and uses of such structures.

BACKGROUND

It is often desirable in many areas of cell biology to "label" a cell with a protein or other moiety at the cell surface. There are a number of systems available to achieve this, such as labelling with biotin or streptavidin, or with gold particles, or with Green Fluorescent Protein (GFP). Integral membrane proteins can also be a useful target for labelling processes.

For example, Armstrong *et al*. (Nat. Commun. (2015) Jun 17;6:7405) described a method of functionalising human mesenchymal stem cells (hMSCs) by polymer-surfactant conjugation of proteins, which enabled delivery of functional proteins to the hMSC membrane. This built on previous work which provided protein-polymer surfactant conjugates (PPSCs) in which surfactant molecules were conjugated to the surface of proteins via electrostatic interactions, either directly (Matsuura *et al*. (1993) *J. Am. Chem. Soc.* vol. 155, 1261-1264), or by way of cationisation of the protein surface by *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide (EDC)-mediated coupling of *N,N′*-dimethyl-1,3-propanediamine (DMPA) groups to solvent-accessible acidic amino acid side chains (Perriman *et al*. (2010) *Nature Chem.* vol. 2, 622-626; Brogan *et al*. (2013) *J. Phys. Chem. B* vol. 117, 8400-8407; Sharma *et al*. (2013) *Adv. Mater.* vol. 25, 2005-2010). This enabled workers to alter the solubility of proteins in water and organic solvent (Matsuura *et al*. (1993) *J. Am. Chem. Soc.* vol. 155, 1261-1264), or to provide proteins in liquid form (as opposed to being in solution; see, for example, Perriman *et al*. (2010) *Nature Chem.* vol. 2, 622-626), or proteins which form a self-standing film (Sharma *et al*. (2013) *Adv. Mater.* vol. 25, 2005-2010).

The present invention described here provides alternative methods for localisation of specific proteins, for the first time including functional enzymes, onto a phospholipid bilayer, such as a cell membrane or the membrane of a liposome.

SUMMARY OF THE INVENTION

According to a first aspect of the invention, there is provided a phospholipid composition which is a phospholipid bilayer or micelle comprising at least one embedded protein-polymer surfactant conjugate, the conjugate comprising an anchor protein, the composition characterised in that the anchor protein is a cationised protein or an anionised protein and is (a) an active enzyme and/or (b) is a protein which does not comprise a

$-CH_2C(O)NCH_3(CH_2)_3N(CH_3)_2H^+$ linker covalently bonded to an amino acid side chain, for example, to an acidic amino acid side chain.

The compositions and methods disclosed herein are applicable to a wide range of cell types including stem cells, lymphocytes and vesicles (including exosomes) and to a very broad range of proteins. The result is a platform with potential in a wide range of clinical and non-clinical applications requiring the targeted delivery of cells to specific locations. Several areas of unmet clinical need can be addressed by use of the invention, for example provision of stem cells for cardiac therapy, cell-based wound glues and organophosphate poisoning treatment.

The presence or absence in a protein of a linker such as that described above (i.e., a $-CH_2C(O)NCH_3(CH_2)_3N(CH_3)_2H^+$ linker), covalently bonded to an amino acid side chain, is readily determined by the skilled person, for example by proteomics methods (such as tryptic digestion followed by mass spectrometry) to determine the amino acid composition of the protein, and therefore to find any unnatural groups such as a linker as described above (for example, amino acid residues modified by DMPA as described elsewhere herein).

The terms "cationised protein" or "anionised protein" indicate that the anchor protein is an electrostatically modified protein. This is a protein which differs from its native state (i.e., from the state of the wild-type version of the protein) in that it has a different surface charge distribution compared to the native (or "unmodified" or "wild-type") protein. Typically, this surface charge distribution is assessed at physiological pH, for example at about pH 6-9, for example about pH 6, 6.5, 7, 7.5, 8, 8.5 or about 9. The native protein may be referred to herein as an "anchor precursor protein". The electrostatic modification differences between the anchor protein and the anchor precursor protein (such as the addition of diamine groups, in some embodiments), as described herein, are present regardless of the pH of the protein environment (e.g., the protein solution). The electrostatic modification may, for example, be achieved by cationisation of an anchor precursor protein, or by anionisation of an anchor precursor protein, or by recombinant expression of a protein having a more positive or a more negative overall charge compared to an anchor precursor protein, for example at physiological pH as described above. The resulting protein may be referred to as a cationised protein in the case of modification to have an overall increased surface positive charge, or as an anionised protein in the case of modification to have an overall increased surface negative charge. Therefore, the anchor protein is not a naturally occurring, or wild-type, protein, for example as determined at physiological pH. For a cationised protein the overall change in surface positive charge may be +1 to +100, for example, +1 to +80, +10 to +70, +20 to +60, or +30 to +50, such as about +5, +6, +7, +8, +9, +10, +11, +12, +13, +14, +15, +16, +17, +18, +19, +20, +21, +22, +23, +24, +25, +26, +27, +28, +29, +30, +31, +32, +33, +34, +35, +36,

+37, +38, +39, +40, +41, +42, +43, +44, +45, +46, +47, +48, +49, +50, +51, +52, +53, +54, or +55. For an anionised protein the overall change in surface negative charge may be -1 to -100, for example, -1 to -80, -10 to -70, -20 to -60, or -30 to -50, such as about -5, -6, -7, -8, -9, -10, -11, -12, -13, -14, -15, -16, -17, -18, -19, -20, -21, -22, -23, -24, -25, -26, -27, -28, -29, -30, -31, -32, -33, -34, -35, -36, -37, -38, -39, -40, -41, -42, -43, -44, -45, -46, -47, -48, -49, -50, -51, -52, -53, -54 or -55.

The name of a particular protein may be used herein to refer interchangeably to an anchor protein or to the anchor protein precursor. For example, the composition may comprise an electrostatically modified thrombin, in which case the term "thrombin" may be used to refer either to the anchor protein or to the anchor precursor protein. Alternatively, a cationised anchor protein may be referred to herein with a "c" prefix, for example, "cThrombin" for cationised thrombin. A recombinantly prepared supercharged protein (as described further below) may be referred to herein with a "sc" prefix, for example, "scOpdA" for supercharged OpdA.

An anchor precursor protein, therefore, as used throughout this specification, is a protein which is modifiable or modified to provide an anchor protein. For example, an anchor precursor protein may be submitted to a chemical method of electrostatic modification, as described elsewhere herein, or is a protein which may be used as a base or starting point for rational design of a modified protein, having an overall charge modified compared to the precursor protein, the modified protein being expressed and obtained using recombinant DNA technology. An anchor precursor protein may, therefore, be a naturally occurring or wild-type protein at physiological pH.

When the anchor protein is an enzyme, it is an active enzyme, i.e. an enzyme which retains the ability to catalyse the reaction catalysed by the anchor precursor protein. For example, the enzyme activity of the anchor protein (being a cationised or anionised anchor precursor protein) may be at least about 75% of the activity of the anchor precursor protein enzyme, for example, at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or at least about 99% the activity of the anchor precursor protein enzyme. Enzyme activity may be determined by any routine method relevant to the enzyme concerned, in accordance with the routine ability of the skilled person. In some cases, the enzyme activity of the anchor protein (being a cationised or anionised anchor precursor protein) may increase compared to the activity of the anchor precursor protein, i.e. have an activity of more than 100% of the activity of the anchor precursor protein.

The phospholipid composition according to the invention may take the form of a bilayer structure, which may form part of a vesicle, liposome, cell, artificial cell or cell organelle, or may take the form of a micelle. The phospholipid composition may, therefore, itself be a vesicle, liposome, artificial cell or cell organelle, or may be a micelle. The term

"composition" does not require the presence of any components other than the phospholipid bilayer or micelle and the embedded protein-polymer surfactant conjugate, although the phospholipid composition may be present within a wider composition such as a cell, a pharmaceutical composition or a surgical composition as mentioned elsewhere herein.

The term "protein-polymer surfactant conjugate", as used throughout this specification, indicates a discrete construct which comprises a protein (referred to as the "anchor protein") having one or more surfactant molecules electrostatically complexed to a charged amino acid residue at the surface of the protein. As mentioned above, the preparation of similar constructs was described, for example, by Perriman *et al*. (2010; *Nature Chem.* vol. 2, 622-626), Brogan *et al*. (2013; *J. Phys. Chem. B* vol. 117, 8400-8407) and Sharma *et al*. (2013; *Adv. Mater.* vol. 25, 2005-2010). The conjugates are proteins having an amphiphilic surfactant corona, as described herein, around at least a portion of the overall structure. The presence of such a corona may be confirmed by comparison of the conjugate with the corresponding wild-type anchor precursor protein, to detect changes in charge and/or size. Techniques such as mass spectrometry, zeta potentiometry, small angle X-ray scattering and/or dynamic light scattering, particularly a combination of two or more of these, may be employed to detect such changes.

The term "embedded" indicates that the protein-polymer surfactant conjugate is located at least partially within the phospholipid bilayer or layer (in the case of a micelle). That is, the protein-polymer surfactant conjugate at least partially intersects with the phospholipid bilayer or layer, rather than merely interacting with a surface of the phospholipid bilayer or layer. Non-embedding/intersecting surface interaction is described, for example, by Futami *et al*. (*J. Biosci. Bioeng.* (2005) vol. 99, 95-103) and such electrostatic interactions between a protein and a phospholipid bilayer or layer are not encompassed by the present invention.

A schematic diagram of a phospholipid composition according to the invention which is a bilayer comprising an embedded protein-polymer surfactant conjugate is shown in Figure 2. A composition according to the invention may be useful to enable the introduction of a wide range of proteins into a wide range of phospholipid bilayer and/or micelle types, such as a cell membrane. Advantageously and surprisingly, the compositions and methods described herein enable the localisation of active enzymes to a cell surface and, thereby, to a tissue or other population of cells.

The anchor protein in the protein-polymer surfactant conjugate may be linked to a secondary molecule which may, for example, also be a protein, or a polypeptide or peptide, or may be one half of a bioconjugation system such as the SpyCatcher/SpyTag system (Reddington & Howarth (2015) *Curr. Op. Chem. Biol*. vol. 29 p94-99; WO2014/176311), or streptavidin/biotin. The secondary molecule may be a protein which is not a cationised or anionised protein and, although forming part of the overall protein-polymer surfactant

conjugate, does not have an amphiphilic surfactant corona. This is because, in the secondary molecule, there is not a sufficiently high surface distribution of charged amino acid side chains to which surfactant molecules may electrostatically complex.

In consequence, in the phospholipid composition, the secondary molecule may be positioned such that it is not embedded with the rest of the protein-polymer surfactant conjugate in the phospholipid bilayer or layer. That is, the secondary molecule may be linked to the anchor protein such that, in the composition, it is positioned to the interior or exterior of a vesicle, liposome, cell, artificial cell, cell organelle, or micelle of which the phospholipid bilayer or layer forms at least a part. A schematic diagram of this arrangement is shown in Figure 3. Effectively, as a result of being part of the overall protein-polymer surfactant conjugate, the secondary molecule is attached to the interior or exterior surface of a vesicle, liposome, cell, artificial cell, cell organelle, or micelle of which the phospholipid bilayer or layer forms at least a part, via linkage to the anchor protein, which is embedded within the phospholipid bilayer or layer. The anchor protein in each protein-polymer surfactant conjugate may be linked to more than one of the secondary molecules as described. Therefore, the term "protein-polymer surfactant conjugate" may encompass any embodiment in which the anchor protein is linked to one or more secondary molecules.

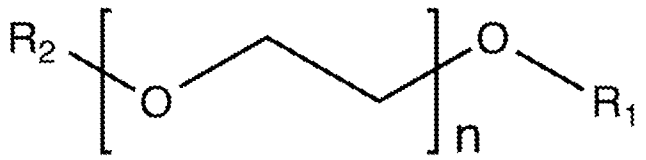
The protein-polymer surfactant conjugate may comprise, by way of non-limiting example, a labelling protein such as GFP, PsmOrange or magnetoferritin, a protein conjugated to labelling molecule or nanoparticle, an enzymatic protein such as a peroxidase or a phosphotriesterase (such as OpdA from *Agrobacterium radiobacter* (SEQ ID NO:10, also described by SEQ ID NO:39) or a functional variant or portion thereof) or a protease (such as thrombin) or an enzyme precursor protein such as prothrombin (e.g., SEQ ID NO:25 or 26)), an adhesion or "homing" protein such as an antibody, lectin, integrin or adhesion molecule (for example the protein CshA from *Streptococcus gordonii* (SEQ ID NO:20), or a functional variant or portion thereof comprising the fibronectin binding domain of CshA (SEQ ID NO:19), or any of the proteins listed in Table 4), a growth factor (such as PlGF-2 (SEQ ID NO:22) or a functional variant or portion thereof comprising PlGF-$2_{(123\text{-}144)}$ (SEQ ID NO:21)), or a carrier protein such as a globin, for example, myoglobin. Depending on the protein, the protein may be cationised or anionised in order to form the anchor protein, or may be the secondary molecule as described above. In addition to the options above, the secondary molecule may be selected from a peptide, polypeptide or other molecule, such as a SpyCatcher or SpyTag motif, biotin or streptavidin.

The term "thrombin" may indicate a thrombin from any species, for example bovine thrombin or human thrombin. The skilled person is readily able to identify suitable thrombin molecules.

The term "surfactant molecule" is well understood by the skilled person, surfactants typically being organic compounds that are amphiphilic, meaning they contain both hydrophobic groups (their tails) and hydrophilic groups (their heads). A cationic surfactant has a positively charged head group, whilst an anionic surfactant has a negatively charged head group. A zwitterionic surfactant is one, such as sodium lauroamphoacetate, which includes both positive and negative charge within the headgroup. The surfactant may additionally comprise functional characteristics such as imaging labels (e.g., a magnetic surfactant (Brown (2013) *Adv. Mater.* vol. 24, 6244-6247) or a fluorescent surfactant), or features such as oxygen binding capability.

The phospholipid composition may comprise lipids other than phospholipids, for example, cholesterol. It may also comprise other components, such as integral membrane proteins. This may especially be the case where the phospholipid composition is a bilayer forming a cell membrane.

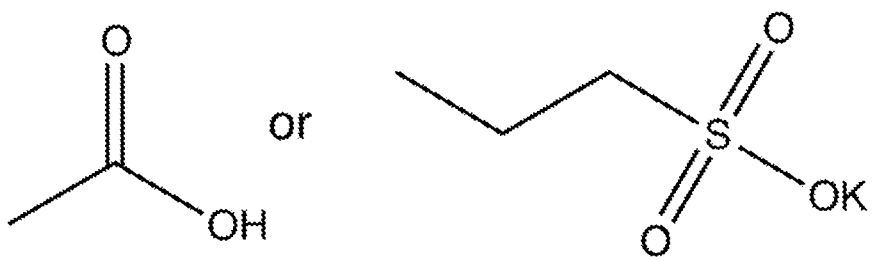
The protein-polymer surfactant conjugate may comprise a polyethylene glycol (PEG)-containing surfactant. For example, the surfactant may have the general structure of Formula I below:



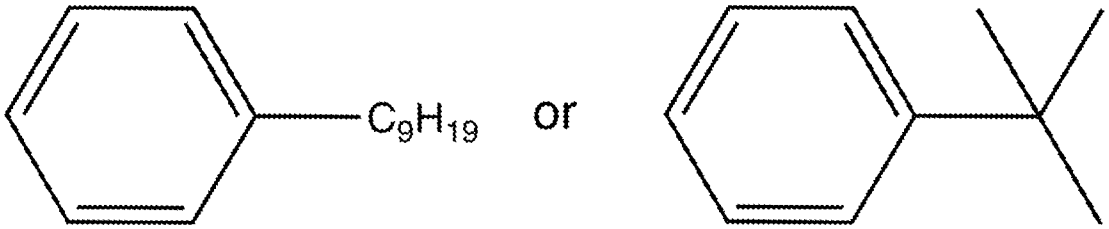
Formula I

In Formula I, n can be any integer including or between 5 and 150, for example any integer including or between 8 and 110. For example, n may be 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 36, 37, 38, 39, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105 or 110.

R_1 may be:

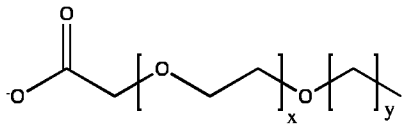


R_2 may be $C_xH_{(2x+1)}$, where x is any integer including or between 8-18; for example, x =may be 11, 12 or 13. R_2 may also be an unsaturated hydrocarbon having 8-18 carbon atoms, for example 11, 12 or 13 carbon atoms. In a further alternative, R_2 may be:

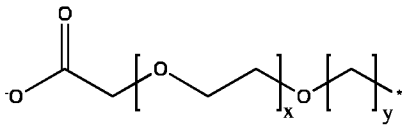


The surfactant may be one of those described herein, such as S621 (Sigma-Aldrich catalogue no. 463221), S907 (Sigma-Aldrich catalogue no. 463256), S1198 (Sigma-Aldrich catalogue no. 473197), or S1783 (oxidised form of glycolic acid ethoxylate 4-nonylphenyl ether, Sigma-Aldrich catalogue no. 238678).

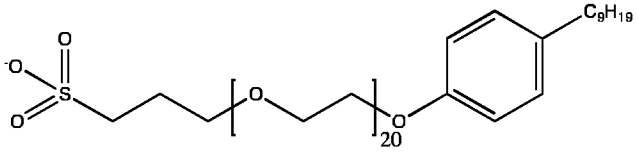
These anionic surfactants have the following structures, as also shown in Figure 4:



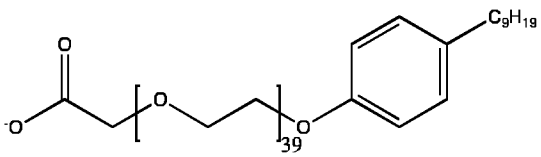
S621



S907



S1198



S1783

For S621 and S907 x = 11-13
For S621, y= 7-9
For S907, y= 14-15

The molecular weight and polydispersity were measured by mass spectrometry and were found to be as follows:

Table 1 - molecular weight and polydispersity of surfactants

Name	**MWt**	**PDi ($Đ_M$)**
S621	621	1.05
S907	907	1.06
S1198	1198	1.03
S1783	1783	1.12

The "polydispersity" reflects the fact that synthetic polymers produced from chemical reactions have a distribution of molecular masses arising from the intrinsically entropic process of polymerisation. The degree of variation is dependent on both the reaction mechanism and the reaction conditions. This degree of variation is defined by the dispersity (*Đ*), which was until recently known as the "polydispersity". It is defined by the equation:

$$Đ_M = M_w/M_n$$

where M_w is the weight-average molar mass and M_n is the number-average molar mass.

The dispersity of a polymer can be estimated using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF).

The protein-polymer surfactant conjugate may comprise a surfactant having a molecular weight of at least about 500 Da, for example, at least about 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000, 2200, 2400, 2600, 2800, 3000, 3200, 3400, 3600, 3800 or at least about 4000 Da.

The protein-polymer surfactant conjugate may comprise a surfactant which is S1783 (i.e., oxidised glycolic acid ethoxylate 4-nonylphenyl ether). Alternatively or additionally, the protein-polymer surfactant conjugate may comprise a cationic surfactant, for example, PEG-15 hydrogenated tallowmodium chloride (sold as Ethoquad® HT25).

The composition according to the invention may comprise a protein-polymer surfactant conjugate which comprises at least two types of surfactant. At least one surfactant may comprise functional characteristics such as imaging labels (e.g., a magnetic surfactant (Brown (2013) *Adv. Mater.* vol. 24, 6244-6247) or a fluorescent surfactant), or features such as oxygen binding capability.

When the anchor protein is an active enzyme, the cationised protein may be obtained by covalent bonding of a cationic or polycationic linker to an acidic amino acid side chain on the protein. For example, this may be achieved by mixing the protein with *N,N'*-dimethyl-1,3-propanediamine (DMPA) or an analogue thereof, in the presence of a carbodiimide such as *N*-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) or dicyclohexyl carbodiimide (DCC). The reaction is shown in Figure 1, which shows that acid residues (numeral (1) in the Figure) are activated towards nucleophilic attack by addition of the zero length cross-linker EDC (2) to form activated o-acylisourea groups (3). The nucleophile DMPA (4) then attacks the activated carbonyl and eliminates isourea to form cationised residues (5). Therefore, the cationised protein may comprise the linker — $CH_2C(O)NCH_3(CH_2)_3N(CH_3)_2H^+$. DMPA or an analogue thereof may be added to the protein prior to mixing with EDC, to ensure the presence of an excess of DMPA or an analogue thereof and thereby avoid cross-linking of proteins to one another.

The step of covalent bonding of a cationic linker to an acidic amino acid side chain on the protein may be carried out in the presence of *N*-hydroxysuccinimide (NHS) or its water-soluble analogue Sulfo-NHS, to improve the stability of electrostatic coupling.

In the present invention, the mixing of the protein with DMPA or an analogue thereof in the presence of a carbodiimide may be allowed to continue for a limited time so as to avoid protein denaturation and/or aggregation. Such a limited time may be, for example, up to or for about 2 hours, or up to or for about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85 or about 90 minutes. Alternatively or additionally, the product of the mixing with DMPA in the presence of a carbodiimide may be subjected to subsequent size exclusion chromatography, with the product from the chromatography being utilised as the cationised protein. The skilled person is capable of determining the theoretical size of the desired anchor protein, so as to determine the appropriate chromatography eluate to collect, for example by use of a calibrated chromatography column. The inclusion in the method of either or both of these method features ensures that the enzyme is retained in an active state. This was not anticipated in view of prior art methods such as those of Armstrong *et al*. (*Nat. Commun*. (2015) Jun 17;6:7405) which, if utilised with an enzyme, would destroy its activity. Suitable methods for preparing an anchor protein are outlined in more detail below.

An analogue of DMPA may be *N,N´*-dimethylhexane-1,6-diamine (DMHA), *N,N´*-dimethylethylenediamine (DMEA), 3-dimethylamino propylamine (DMAPA), ethylenediamine (EN), 1,3-diaminopropane (DAP), 1,4-diaminobutane (DAB), 1,5-diaminopropane (DAP), 1,6-diaminohexane (DAH), hexamethylenediamine (HMA), 1,7-diaminheptane (DAH) 1,8-diaminooctane (DAO) and 2-(2-aminoethyl)guanidine (AEG). Other suitable nucleophiles may be contemplated by the skilled person, for example, charged nucleophiles. For example, nucleophiles could also include other primary, secondary and tertiary alkyl diamines and alkyl diamines terminated with a quaternary amine if the opposing terminus contains either a primary, secondary or tertiary amine. Polyalkylamines such as polyethylenimine as either linear chains or branched structures are also contemplated.

Alternatively, the electrostatically modified protein may be obtained by anionisation of the protein. This may be achieved, for example, by nucleophilic addition of dicarboxylic acids (HOOC-R-COOH) to lysine side-chains of the native proteins.

In a further alternative, the electrostatically modified cationised or anionised protein (which does not comprise a $-CH_2C(O)NCH_3(CH_2)_3N(CH_3)_2H^+$ linker covalently bonded to an amino acid side chain) may be obtained by recombinant expression of an anchor protein having an altered charge, i.e., a more positive or a more negative overall charge compared to an anchor precursor protein. Recombinant modification may comprise recombinantly expressing an anchor protein which is a mutant comprising one or more amino acid

substitutions within its overall amino acid sequence compared to the sequence of the non-mutant anchor precursor protein, the amino acid substitutions introducing a different surface charge distribution to the anchor protein compared to the anchor precursor protein, by providing a different amino acid charge to the native amino acid at the or each substitution position. Such proteins are known in the art and are referred to as "supercharged" proteins.

For example, an amino acid having an uncharged side group may be replaced by an amino acid having a positively or negatively charged side group (to give an overall charge change of +1 or -1 respectively), or an amino acid having a negatively charged side group may be replaced by an amino acid having a positively charged side group (to give an overall charge change of +2), or an amino acid having a positively charged side group may be replaced by an amino acid having a negatively charged side group (to give an overall charge change of -2), provided that the tertiary structure and/or biological activity of the protein is not significantly altered. This rational design approach may be especially advantageous if the function/activity of the protein depends on the involvement of a particular amino acid, for example one having a charged side group, since the user can direct protein surface charge alterations to non-critical amino acid positions; this may not always be possible with the chemical modification methods described elsewhere herein. The biological activity of the protein in native (anchor precursor protein) or supercharged (anchor protein) form may be determined using an assay appropriate for the protein, as readily selected by the skilled person. For example, thrombin activity may be assessed by contacting with fibrinogen and detecting the rate of fibrin formation. Specific such assays are described in the detailed methods below.

Typically, the amino acid sequence identity, determined at a global level (otherwise known as "global sequence identity"), between the native protein (i.e., the anchor precursor protein) and the recombinantly modified protein (i.e., the anchor protein) is at least about 60%, for example at least about 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or at least about 99%. Determination of sequence identity at a global level may be carried out using, for example, the Needleman-Wunsch Global Sequence Alignment Tool available on the internet via the NCBI Blast® internet site (blast.ncbi.nlm.nih.gov/Blast.cgi). This tool allows a user to compare two sequences across their entire span.

For example, where the protein is CshA (SEQ ID NO:20) or a functional variant or portion thereof, it may be a protein which comprises the amino acid sequence SEQ ID NO:19 (which is the fibronectin-binding portion of CshA), or an amino acid sequence which is at least about 90% identical, at a global level, to SEQ ID NO:19. Where the protein is a variant of CshA, the global sequence identity of the variant with CshA (SEQ ID NO:20) may be less

than 60%, provided that the variant comprises an amino acid sequence which is at least 90% identical, at a global level, to SEQ ID NO:19, optionally at least about 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or at least about 99%, or which is SEQ ID NO:19.

In an alternative, where the protein is PlGF-2 (SEQ ID NO:22) or a functional variant or portion thereof, the protein comprises SEQ ID NO:21 (which is PlGF-$2_{(123-144)}$), or an amino acid sequence which is at least about 90% identical, at a global level, to SEQ ID NO:21. Where the protein is a variant of PlGF-2, the global sequence identity of the variant with PlGF-2 (SEQ ID NO:22) may be less than 60%, provided that the variant comprises an amino acid sequence which is at least 90% identical, at a global level, to SEQ ID NO:21, optionally at least about 91%, 92%, 93%, 94% or at least about 95%, or which is SEQ ID NO:21.

Where the protein is OpdA (SEQ ID NO:10 or SEQ ID NO:39) or a functional variant or portion thereof, the protein comprises the native amino acids at positions 31, 33, 145, 177, 206 and 277 as found in SEQ ID NO:10 or SEQ ID NO:39, i.e., histidine at positions 31, 33, 177 and 206, carboxylated lysine at position 145 and aspartic acid at position 277. (SEQ ID NO:10 is the sequence showing "lysine" at position 145, which the skilled person understands to be a carboxylated lysine. SEQ ID NO:39 is identical to SEQ ID NO:10 other than explicitly specifying carboxylated lysine at position 145.) Therefore, this means that the amino acids at these positions are not substituted by another amino acid and that any variant sequence comprises these positions. For example, a functional variant of SEQ ID NO:10 may comprise a portion of SEQ ID NO:10 which is at least about 90% identical to a sequence having (i.e., comprising) amino acids 31-277 of SEQ ID NO:10 (for example, amino acids 30-280, 25-285, 20-290, 15-295, 10-300, 5-305), the portion comprising histidine at positions 31, 33, 177 and 206, carboxylated lysine at position 145 and aspartic acid at position 277. A functional variant of SEQ ID NO:39 may comprise a portion of SEQ ID NO:39 which is at least about 90% identical to a sequence having (i.e., comprising) amino acids 31-277 of SEQ ID NO:39 (for example, amino acids 30-280, 25-285, 20-290, 15-295, 10-300, 5-305), the portion comprising histidine at positions 31, 33, 177 and 206, carboxylated lysine at position 145 and aspartic acid at position 277. These amino acid positions in OpdA are known to be important to the correct functioning of the enzyme.

For the avoidance of doubt, SEQ ID NO:39 describes the same protein sequence as SEQ ID NO:10, merely providing an explicit indication of carboxylated lysine at position 145, which would in any case be understood by the skilled person to be present in SEQ ID NO:10.

Reference throughout this specification to a "functional variant" indicates an amino acid sequence which is not identical to the non-variant sequence, but which displays activity which is not significantly reduced (e.g., is substantially similar) compared to the activity of the non-variant sequence. For example, a non-variant sequence may be an enzyme having

a level of activity which may be assessed by the skilled person, whereas the functional variant retains a level of activity of at least 75%, preferably at least about 80%, 85%, 90% or at least about 95% compared to the non-variant sequence. In some cases, the activity of the functional variant may be greater than the activity of the non-variant. Alternatively, the non-variant sequence may be capable of binding another entity (such as a molecule, protein, peptide, antigen or cell) and the binding affinity may be assessed by the skilled person. A functional variant retains a level of binding affinity of at least 75%, preferably at least about 80%, 85%, 90% or at least about 95% compared to the non-variant sequence, or may have an increased binding affinity. The skilled person is readily able to determine whether a variant amino acid sequence is a functional variant or not.

Such recombinant methods can be used to prepare an anchor protein which is electrostatically modified and which does not comprise a $-CH_2C(O)NCH_3(CH_2)_3N(CH_3)_2H^+$ linker covalently bonded to an amino acid side chain. Typically, such an anchor protein consists of amino acids which are naturally occurring, for example which are selected from proteinogenic amino acids (including canonical amino acids) or non-proteinogenic amino acids. A proteinogenic amino acid is one which is incorporated into proteins by natural translation processes. A non-proteinogenic amino acid is one which is not utilised in natural protein translation but which may be incorporated into an amino acid sequence by a mechanism which may include natural or artificial post-translational mechanisms. Non-limiting examples of amino acids which may be included within the anchor protein are provided in Tables 2 and 3 below:

Alanine	Phenylalanine	Glutamine	**Arginine**	Selenocysteine
Isoleucine	Tryptophan	Serine	**Histidine**	Pyrrolysine
Leucine	Tyrosine	Threonine	**Lysine**	
Methionine	Asparagine	*Aspartic acid*	Glycine	
Valine	Cysteine	*Glutamic acid*	Proline	

Table 2: examples of proteinogenic amino acids; bold indicates positively charged amino acids, italic indicates negatively charges amino acids.

Modifications of proteinogenic and non-proteinogenic amino acids are also contemplated, provided that they do not include a $-CH_2C(O)NCH_3(CH_2)_3N(CH_3)_2H^+$ linker covalently bonded to an amino acid side chain.

Non-naturally occurring amino acids may also be included (such as those which may be introduced into a protein by use of a unique codon and a corresponding aminoacyl-tRNA system), provided that any such amino acid does not comprise a $-CH_2C(O)NCH_3(CH_2)_3N(CH_3)_2H^+$ linker covalently bonded to an amino acid side chain.

β-alanine	γ-aminobutyric acid	δ-aminovulinic acid	4-aminobenzoic acid	aminoisobutyric acid
dehydroalanine	cystathione	lanthionine	djenkolic acid	diaminopimelic acid
α-amino-n-butyric acid	norvaline	norleucine	alloisoleucine	t-leucine
α-amino-n-heptanoic acid	pipecolic acid	α,β-diaminopropionic acid	α,γ-diaminobutyric acid	ornithine
allothreonine	homocysteine	homoserine	β-amino-n-butyric acid	β-aminoisobutyric acid
γ-aminobutyric acid	α-aminoisobutyric acid	isovaline	sarcosine	N-ethyl glycine
N-propyl glycine	N-isopropyl glycine	N-methyl glycine	N-ethyl glycine	N-ethyl alanine
N-methyl β-alanine	N-ethyl β-alanine	isoserine	α-hydroxy-γ-aminobutyric acid	homonorleucine
tellurocysteine	telluromethionine	ornithine	citrulline	γ-carboxyglutamate
hydroxyproline	hypusine	pyroglutamic acid		

Table 3: examples of non-proteinogenic amino acids

Typically, an anchor protein within the protein-polymer surfactant conjugate may comprise a percentage of positively charged amino acid residues (such as those marked bold in Table 2 above), determined as a percentage of the total number of amino acid residues in the protein, which is greater than the percentage of such residues in the corresponding anchor precursor protein. For example, the anchor precursor protein may have 5.0-17.5% of its total amino acid residues as positively charged residues and the anchor protein may have a higher percentage than in the corresponding anchor precursor protein. The anchor protein may have at least about 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29% or at least about 30% of its total amino acid residues as positively charged residues. For example, the supercharged GFP described herein has about 28% of its total amino acid residues as positively charged residues whilst the supercharged OpdA described herein has about 18% of its total amino acid residues as positively charged residues. Non-supercharged naturally occurring OpdA has 13% of its total amino acid residues as positively charged residues, whilst non-supercharged GFP has 15% of its total amino acid residues as positively charged residues. The overall charge of the protein may typically be assessed at physiological pH, as described above.

The anchor protein may comprise only amino acids selected from the group consisting of alanine, isoleucine, leucine, methionine, valine, phenylalanine, tryptophan, tyrosine, asparagine, cysteine, glutamine, serine, threonine, aspartic acid, glutamic acid, glycine, proline, selenocysteine and pyrrolysine. Alternatively or additionally, the anchor protein may comprise fewer arginine and/or histidine and/or lysine residues than the number of arginine and/or histidine and/or lysine residues present in the corresponding anchor precursor protein. For example, the anchor protein may comprise no arginine and/or no histidine and/or no lysine residues. Alternatively or additionally, the anchor protein may comprise fewer positively charged residues than the corresponding anchor precursor protein, or may comprise fewer negatively charged residues than the corresponding anchor precursor protein. One or more uncharged and/or positively charged residues in the anchor precursor protein each may be replaced by a negatively charged residue, to form an anionised anchor protein. Alternatively, one or more uncharged and/or negatively charged residues in the anchor precursor protein each may be replaced by a positively charged residue, to form a cationised anchor protein.

Examples of the production of such a modified ("supercharged") protein, in the context of Green Fluorescent Protein (GFP), are disclosed in Lawrence *et al*. (*J. Am. Chem. Soc*. (2007) vol. 129 p.10110-10112). Such so-called "supercharged" proteins have previously been used to facilitate delivery of molecules through the phospholipid bilayer cell membrane to the interior of a cell (Zang *et al*. (2017) *PLoS One* 12(6):e0180138; WO2009/134808; WO2010/129023; WO2016/069910; Thompson *et al*. (2012) *Methods Enzymol*. vol. 503 p. 293-319; McNaughton *et al*. (2009) *Proc. Natl. Acad. Sci. U.S.A*. vol. 106 p. 6111-6116). It is, therefore, wholly surprising that, when incorporated into a protein-polymer surfactant conjugate as described herein, a phospholipid composition, such as a cell, comprising an embedded protein-polymer surfactant conjugate can be obtained.

In a composition according to the invention and as mentioned above, the phospholipid bilayer may form the surface membrane of a cell. The cell may be any comprising a phospholipid bilayer, particularly a cell which does not also comprise an exterior cell wall. However, in this specification, the term "cell" encompasses a protoplast or spheroplast, i.e., a cell normally comprising a cell wall but having had at least some of said wall removed or disrupted, for example, by a mechanical or enzymatic process.

The cell may be any which a user desires to contact with a protein-polymer surfactant conjugate in order to embed the protein-polymer surfactant conjugate in the cell membrane, such as an animal or a plant cell, or a microorganism cell, for example a mammalian cell *in vivo* or *in vitro* or *ex vivo* such as in cell or tissue culture. The mammalian cell may be a human, dog, cat or horse cell, or a bovine, porcine or ovine cell.

For example, the mammalian cell may be a human cell including a mesenchymal stem cell, or a cell derived from an embryonic stem cell or an induced pluripotent stem cell, which may be a human cell. The cell may be one which is not a human cell or human embryonic cell or human embryonic stem cell and/or is not derived from a human cell or human embryonic cell or human embryonic stem cell. The cell may also be a specialised cell such as a cardiomyocyte for targeting the heart, a chondrocyte for targeting cartilage, an osteoblast for targeting bone, a hepatocyte for targeting liver, a pancreatic islet beta cell for targeting the pancreas, a nerve cell or neural progenitor cell for targeting the brain, an endothelial cell for targeting the internal lumen of blood vessels, a myocyte for targeting muscles or a ligamentocyte for targeting ligaments. These examples are not limiting and any specialised cell might be used for any part of the body. In addition, cell lines such as CHO or HELA might be used for animal studies or *in vitro* studies to demonstrate cell distribution using an appropriate label.

The composition according to the invention may be one wherein the phospholipid composition forms at least a portion of the membrane of a mesenchymal stem cell (i.e., the cell membrane of the MSC is the phospholipid composition). The stem cell may be one which has not been obtained from a human embryonic cell or stem cell. The phospholipid composition may comprise one or more of:

supercharged fibronectin-binding domain of CshA;
supercharged CshA, or a supercharged functional variant or portion thereof comprising fibronectin-binding domain of CshA;
supercharged PlGF-$2_{(123-144)}$, or supercharged PlGF-2 or a supercharged functional variant or portion thereof comprising PlGF-$2_{(123-144)}$;
cationised or supercharged prothrombin or thrombin, or a cationised or supercharged functional variant of prothrombin or thrombin.

The phospholipid composition of the invention may form at least a portion of the membrane of a mesenchymal stem cell or a cardiomyocyte. In this context, the phospholipid composition may comprise supercharged fibronectin-binding domain of CshA, or supercharged CshA or a supercharged functional variant or portion of CshA comprising the fibronectin-binding domain.

When the phospholipid bilayer forms the surface membrane of a cell, the protein-polymer surfactant conjugate may be embedded in the phospholipid bilayer for 1-30 days, or 1-15 or 1-10 or 1-5 days after the phospholipid composition according to the invention is formed, by contacting the cell with the protein-polymer surfactant conjugate. For example, the protein-polymer surfactant conjugate may be embedded for about 1, 2, 3, 4, 5, 6, 7, 8, 9 or about 10 days.

The phospholipid composition according to the invention may be present within a complex composition further comprising at least one additional component, for example, water, a buffer solution, one or more components required to form a pharmaceutical composition as described below, or one or more components required to form a surgical composition such as a liquid or a scaffold material such as a membrane or a fabric.

According to a second aspect of the invention, there is provided a method of preparing a phospholipid composition according to the first aspect of the invention, the method comprising

a) providing a protein-polymer surfactant conjugate; and

b) contacting a phospholipid bilayer or micelle with the conjugate;

wherein the protein-polymer surfactant conjugate comprises an anchor protein which is a cationised protein or an anionised protein and (i) is an active enzyme and/or (ii) is a protein which does not comprise a $-CH_2C(O)NCH_3(CH_2)_3N(CH_3)_2H^+$ linker covalently bonded to an amino acid side chain, for example, to an acidic amino acid side chain.

The phospholipid bilayer may be in the form of a cell, artificial cell, liposome or other vesicle, or may form a part of a cell, artificial cell, liposome or other vesicle. In a method according to the second aspect of the invention, the phospholipid bilayer may be a cell which is contacted in step (b) with a protein-polymer surfactant conjugate as defined above and incubated at a temperature of at least about 10°C for a period of at least about 2 minutes. The temperature may typically be about 30-40°C, for example about 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 or about 40°C, for example about 37°C ± about 1°C. The time period may typically be 2-60 minutes, for example about 2, 3, 4, 5, 10, 15, 20, 30, 40, 50 or about 60 minutes, for example about 15, about 20 or about 30 minutes. The step may take place in an atmosphere of about 0-10% CO_2, for example, of about 5% CO_2. When the phospholipid bilayer is in the form of an artificial cell, liposome or other non-cell vesicle, step (b) may be conducted at room temperature (e.g., between about 15°C and about 25°C) with <1% CO_2, for example, in air.

Step (b) of the method according to the second aspect of the invention may optionally followed by a step (c) of washing the phospholipid bilayer or micelle (e.g. a cell), for example using a buffer such as Phosphate Buffered Saline (PBS), for example with two or more washing steps. The skilled person is readily able to adapt such steps as required, and to determine when a washing step is desirable.

Step (a) of the method according to the second aspect of the invention, to provide a protein-polymer surfactant conjugate, comprises contacting an anchor protein which is a cationised protein or an anionised protein with a surfactant under conditions which enable

electrostatic conjugation of the surfactant with the protein. The surfactant may be added in solid or liquid form to a solution of the protein. The surfactant may be added in an amount equivalent to 0.5-5 moles surfactant per cationic site on the protein, for example, equivalent to about 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9 or about 3.0 moles surfactant per cationic site on the protein. The protein may be in a solution with a suitable buffer such as a HEPES buffer, with or without $CoCl_2$, or in a Tris-HCl buffer. The selection of an appropriate buffer is within the routine abilities of the skilled person. The conditions may include a pH of between 5 and 8, for example of about 5, 6, 7 or about 8 (encompassing any individual intermediate pH value between 5.1 and 5.9, between 6.1 and 6.9, and between 7.1 and 7.9), and may include agitation of the mixture for 0-30 hours, for example, for about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or about 12 hours, at a temperature of 0-25°C, for example at about 4°C or at about room temperature. For example, the conjugation conditions as described by Armstrong *et al*. (*Nat. Commun*. (2015) Jun 17;6:7405) may be suitable.

A "cationic site" is a position within the amino acid sequence of the protein which has an amino acid with a positively charged side chain or comprising a cationic (i.e., positively charged) linker. The number of cationic sites within an anchor protein may be determined without use of inventive skill by the skilled person.

The surfactant may comprise polyethylene glycol, which may, for example, have a molecular weight of at least about 500 Da, for example, at least about 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000, 2200, 2400, 2600, 2800, 3000, 3200, 3400, 3600, 3800 or at least about 4000 Da. The surfactant may be in a buffer solution at a concentration of 5-50mg/mL, for example, about 10, 15, 20, 25, or about 30mg/mL.

The surfactant may be S1783 (i.e., oxidised glycolic acid ethoxylate 4-nonylphenyl ether). Alternatively, the surfactant conjugate may comprise a cationic surfactant, for example, PEG-15 hydrogenated tallowmodium chloride (sold as Ethoquad® HT25).

The anchor protein may be linked to a secondary molecule, as described above, prior to contacting with the surfactant.

Step (a) may also comprise, prior to contacting the phospholipid bilayer or micelle with the protein-polymer surfactant conjugate, a buffer exchange step. The buffer exchange step may comprise a spin concentration of the product of the step of contacting the cationised or anionised protein with the surfactant. Alternatively, the buffer exchange step may comprise a dialysis step. Such methods are described in the Examples section herein and are within the routine ability of the skilled person.

When the anchor protein is an active enzyme, it may be a cationised protein which has been obtained by covalent bonding of a cationic linker (which may be polycationic) to an acidic

amino acid side chain on the protein. For example, at least one acidic amino acid side chain may comprise a $-CH_2C(O)NCH_3(CH_2)_3N(CH_3)_2H^+$ linker. This may be achieved by a method in which a solution of *N,N'*-dimethyl-1,3-propanediamine (DMPA) or an analogue thereof is mixed with an anchor precursor protein (as defined above), in the presence of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC). As outlined above, the reaction is shown in Figure 1. An analogue of DMPA may be *N,N´*-dimethylhexane-1,6-diamine (DMHA), *N,N´*-dimethylethylenediamine (DMEA), 3-dimethylamino propylamine (DMAPA), ethylenediamine (EN), 1,3-diaminopropane (DAP), 1,4-diaminobutane (DAB), 1,5-diaminopropane (DAP), 1,6-diaminohexane (DAH), hexamethylenediamine (HMA), 1,7-diaminheptane (DAH) 1,8-diaminooctane (DAO) and 2-(2-aminoethyl)guanidine (AEG). Other suitable nucleophiles may be contemplated by the skilled person, for example, charged nucleophiles. For example, nucleophiles could also include other primary, secondary and tertiary alkyl diamines and alkyl diamines terminated with a quaternary amine if the opposing terminus contains either a primary, secondary or tertiary amine. Polyalkylamines such as polyethylenimine as either linear chains or branched structures are also contemplated.

Therefore, when the anchor protein is an active enzyme, it may be obtained by a method comprising:

i) mixing a solution of an anchor precursor protein with a pH-neutralised solution of N,N'-dimethyl-1,3-propanediamine (DMPA) or analogue thereof and optionally (for example, if step (ii) is conducted non-concurrently with step (i)) adjusting the mixture to pH 5-7, for example about pH 6;

ii) subsequently or concurrently adding a carbodiimide such as N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and adjusting the mixture to pH 4-7;

iii) agitating the mixture from (ii) for 1-30 hours at pH 4-7, at a temperature of 0-25°C;

iv) dialysing the protein in the mixture from (iii) against water or buffer for at least 4 hours at pH 6.5-8.5;

v) if necessary, adjusting the pH of the mixture from (iv) to pH 6.5-8.5.

In the method, either step (iii) continues for no longer than about 120 minutes, for example, for no longer than about 90 minutes; and/or the method further comprises a step (vi) of conducting size exclusion chromatography on the mixture from step (iv), or from step (v) when present, and obtaining an eluate comprising an anchor protein at the required molecular weight. Either or both of these limitations ensure that the process is controlled to reduce or prevent protein denaturation and/or aggregation, such that the anchor protein

enzyme product of the method retains enzymatic activity. This was not predictable from the disclosure of the prior art.

The solution of anchor precursor protein used in step (i) may be prepared in any conventional buffer, for example, HEPES. The anchor precursor protein solution is mixed with DMPA at a ratio of moles DMPA : number of anionic sites on the protein of 100:1 - 400:1, for example, about 100:1, 150:1, 200:1, 250:1, or about 300:1. EDC is added at a ratio of moles EDC : number of anionic sites on the protein of 30:1 - 60:1, for example, about 30:1, 31:1, 32:1, 33:1, 34:1, 35:1, 40:1, 45:1 or about 50:1.

An "anionic site" is a position within the amino acid sequence of the protein which has an amino acid with a negatively charged side chain. The number of anionic sites within an anchor precursor protein may be determined using the routine ability of the skilled person.

Step (ii) may be completed at the same time as step (i), i.e. the protein solution, DMPA and EDC may be mixed concurrently. Where step (ii) is completed after step (i), step (ii) may be a single step as defined above and immediately followed by step (iii), or may be subdivided into two steps (iia) in which a portion of the EDC is added to the mixture from step (i) and the mixture agitated for about 2, 3, 4, 5, 6, 7 or about 8 hours at a temperature of 0-25°C, followed by (iib) in which further EDC is added to the mixture from (iia) and the agitation continues; step (iib) is followed by step (iii).

The required agitation in step (iii) may be achieved by any conventional means such as stirring, for example, and the pH may be about 4, about 5, about 6 or about 7 (encompassing any intermediate pH value between 4.1 and 4.9 and between 5.1 and 5.9 and between 6.1 and 6.9). When the time period in step (iii) exceeds 120 minutes, it may continue for about 20-30 hours, for example, about 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or about 30 hours, for example about 24 hours. All steps may, for example, be conducted at about room temperature, for example, 18-23°C, or may be conducted at about 4°C.

The skilled person may determine the appropriate time period for step (iii), whether or not there is a subsequent size exclusion chromatography step, by conducting the step (iii) for a range of time periods and testing for the retention of enzyme activity, using any routine suitable assay depending on the enzyme, to determine the optimal time period for step (iii).

An example of a specific method for providing a cationised OpdA anchor protein for inclusion in a protein-polymer surfactant conjugate is as follows:

1) adding a solution of OpdA (in 30mM HEPES with 100μM $CoCl_2$) to a pH neutralised solution of DMPA at a ratio of DMPA to anionic sites in the OpdA of 300:1 and adjusting the pH to about 5.1;

2) adding EDC to the mixture from (1), at a ratio of moles of EDC to anionic sites in the OpdA of 50:1, in two half-additions 4 hours apart;

3) agitating the mixture from (2) for a total of about 20 hours (including the period where only the first half-addition of EDC is included) at a temperature of about 4°C;

4) desalting the mixture from (3) using 10,000 MWCO spin concentrators;

5) conducting size exclusion chromatography on the mixture from (4) and retaining eluate comprising the cationised OpdA anchor protein.

Step (4) may be repeated about 1, 2, 3, 4, 5 or about 6 times.

An example of a specific method for providing a cationised thrombin anchor protein for inclusion in a protein-polymer surfactant conjugate is as follows:

01) adding a solution of Thrombin (in 60mM HEPES) to a pH neutralised solution of DMPA at a ratio of DMPA to anionic sites in the Thrombin of 150:1 and adjusting the pH to about 6.5;

02) adding EDC to the mixture from (01), at a ratio of moles of EDC to anionic sites in the Thrombin of 34:1;

03) agitating the mixture from (02) for about 60 minutes at room temperature;

04) diluting the mixture from (03) with 20mM HEPES (pH7), for example about 4-fold, at 4°C and applying to a 10K MWCO spin concentrator.

Step (04) may be repeated about 1, 2, 3, 4, 5 or about 6 times.

In an alternative general method, the protein-polymer surfactant conjugate may be prepared by contacting an anchor protein which is an anionised protein as described with a surfactant which is a cationic surfactant. For example, the protein may be anionised by nucleophilic addition of dicarboxylic acids (HOOC-R-COOH) to the lysine side-chains of the native protein.

Alternatively or additionally to the above modifications, the anchor protein to be contacted with a surfactant as described above, i.e., for inclusion in the protein-polymer surfactant conjugate provided for use in the method according to the second aspect of the invention, may have been obtained by a recombinant method, to provide an anionised anchor protein or cationised anchor protein which does not comprise a $-CH_2C(O)NCH_3(CH_2)_3N(CH_3)_2H^+$ linker covalently bonded to an amino acid side chain, for example, to an acidic amino acid side chain. For example, the anchor protein may be obtained by a method comprising

expression of a recombinant DNA sequence encoding for a supercharged protein. The resulting protein, which is the anchor protein, subsequently may be isolated.

For example, preparation of a supercharged protein may involve substituting an amino acid having an uncharged side group with an amino acid having a charged side group, or substituting an amino acid with a charged side group with a side group having the opposite charge, provided that the tertiary structure and/or biological activity of the protein is not significantly altered. This may be especially advantageous if the function/activity of the protein depends on the involvement of an amino acid with a charged side group, since the user can direct protein surface charge alterations to non-critical amino acid positions. Where the protein is an enzyme or a protein having another biological activity, the supercharged protein may comprise a functionally important portion or domain of the protein in wild-type form, i.e., the domain or portion may not include any amino acid substitutions. Alternatively or additionally, the skilled person may establish, from the literature or using routine methods, a wild-type amino acid residue at one or more positions which is critical to protein activity (e.g., enzymatic activity); the supercharged protein may comprise the wild-type amino acid at the or each position, with amino acids at other positions optionally being substituted.

Typically, the amino acid sequence identity, determined at a global level (otherwise known as "global sequence identity"), between the recombinantly modified supercharged protein (i.e., the anchor protein) and the native protein (i.e., the anchor precursor protein) is at least about 60%, for example at least about 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or at least about 99%. Determination of sequence identity at a global level may be carried out using, for example, the Needleman-Wunsch Global Sequence Alignment Tool available on the internet via the NCBI Blast® internet site (http://blast.ncbi.nlm.nih.gov/Blast.cgi). As mentioned above, the sequence identity of a functionally important domain may be at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or at least about 99% identical between the anchor protein and the anchor precursor protein.

Such recombinant methods can be used to prepare an anchor protein which is electrostatically modified relative to the anchor precursor protein and which does not comprise a $-CH_2C(O)NCH_3(CH_2)_3N(CH_3)_2H^+$ linker covalently bonded to an amino acid side chain. Typically, such an anchor protein consists of amino acids which are naturally occurring, for example which are selected from proteinogenic amino acids (including canonical amino acids) or non-proteinogenic amino acids, as described in more detail above. Modifications of proteinogenic and non-proteinogenic amino acids are also contemplated, provided that they do not include a $-CH_2C(O)NCH_3(CH_2)_3N(CH_3)_2H^+$ linker covalently bonded

to an amino acid side chain. Non-naturally occurring amino acids may also be included, as described above.

Typically, an anchor protein for inclusion in the protein-polymer surfactant conjugate for use in the method according to the second aspect of the invention may comprise a percentage of positively charged amino acid residues (such as those marked bold in Table 2 above), determined as a percentage of the total number of amino acid residues in the protein, which is greater than the percentage of such residues in the corresponding anchor precursor protein. For example, the anchor precursor protein may have 5.0-17.5% of its total amino acid residues as positively charged residues and the anchor protein may have a higher percentage than in the corresponding anchor precursor protein. The anchor protein may have at least about 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29% or at least about 30% of its total amino acid residues as positively charged residues. For example, the supercharged GFP described herein has about 28% of its total amino acid residues as positively charged residues whilst the supercharged OpdA described herein has about 18% of its total amino acid residues as positively charged residues. Non-supercharged naturally occurring OpdA has 13% of its total amino acid residues as positively charged residues, whilst non-supercharged GFP has 15% of its total amino acid residues as positively charged residues. The overall charge of the protein may typically be assessed at physiological pH as described above.

The anchor protein may comprise only amino acids selected from the group consisting of alanine, isoleucine, leucine, methionine, valine, phenylalanine, tryptophan, tyrosine, asparagine, cysteine, glutamine, serine, threonine, aspartic acid, glutamic acid, glycine, proline, selenocysteine and pyrrolysine. Alternatively or additionally, the anchor protein may comprise fewer arginine and/or histidine and/or lysine residues than the number of arginine and/or histidine and/or lysine residues present in the corresponding anchor precursor protein. For example, the anchor protein may comprise no arginine and/or no histidine and/or no lysine residues. Alternatively or additionally, the anchor protein may comprise fewer positively charged residues than the corresponding anchor precursor protein, or may comprise fewer negatively charges residues than the corresponding anchor precursor protein. One or more uncharged and/or positively charged residues in the anchor precursor protein each may be replaced by a negatively charged residue, to form the anchor protein. Alternatively, one or more uncharged and/or negatively charged residues in the anchor precursor protein each may be replaced by a positively charged residue, to form the anchor protein.

Examples of the production of such a modified ("supercharged") protein, in the context of Green Fluorescent Protein (GFP), are disclosed in Lawrence *et al*. (*J. Am. Chem. Soc*. (2007) vol. 129 p.10110-10112). The methods disclosed therein are readily adaptable by the

skilled person to enable provision of other supercharged proteins, for example as described herein.

The recombinant DNA may comprise SEQ ID NO:2, or any polynucleotide sequence encoding for supercharged OpdA, for example, encoding for SEQ ID NO:11, which is a supercharged OpdA having carboxylated lysine at the position equivalent to position 145 in SEQ ID NO:10; this position is position 151 in SEQ ID NO:11. SEQ ID NO:40 is the same sequence as SEQ ID NO:11, explicitly indicating that carboxylated lysine is present at position 151, as would be understood by the skilled person to be the case in SEQ ID NO:11. Therefore, SEQ ID NO:2 may also be said to encode for SEQ ID NO:40.

The recombinant DNA sequence may encode for a fusion protein comprising the supercharged protein (which may be the anchor protein as described herein) and a secondary molecule. The secondary molecule may be, or may comprise, by way of non-limiting example, a labelling protein such as GFP, PsmOrange or magnetoferritin, a protein conjugated to labelling molecule or nanoparticle, an enzymatic protein such as a peroxidase or a phosphotriesterase (such as OpdA from *Agrobacterium radiobacter*) or a protease (such as thrombin), or an enzyme precursor protein such as prothrombin, an adhesion or "homing" protein such as an antibody, lectin, integrin or adhesion molecule (for example the protein CshA from *Streptococcus gordonii*), or a functional variant or portion of CshA comprising the fibronectin binding domain of CshA), a growth factor such as PlGF-2 or a portion thereof or functional variant thereof comprising PlGF-$2_{(123-144)}$, or a carrier protein such as a globin, for example, myoglobin, or a peptide or polypeptide such as a SpyCatcher or SpyTag motif.

The anchor protein may be supercharged GFP (e.g. SEQ ID NO:12) or supercharged OpdA (SEQ ID NO:11, also described by SEQ ID NO:40). Alternatively or additionally, the secondary molecule included within the fusion protein may be a thrombin or a prothrombin (e.g., SEQ ID NO:25 or 26), CshA (e.g., SEQ ID NO:20, from *Streptococcus gordonii*) or a functional variant or portion thereof comprising the fibronectin binding domain (SEQ ID NO:19), OpdA (SEQ ID NO:10, from *Rhizobium radiobacter*, or a functional variant or portion thereof; this sequence is also described as SEQ ID NO:39), Placental Growth Factor-2 (SEQ ID NO:22), or a functional variant or portion thereof comprising PlGF-$2_{(123-144)}$ (SEQ ID NO:21), a SpyCatcher polypeptide (SEQ ID NO:23) or a SpyTag polypeptide (SEQ ID NO:24). Therefore, the secondary molecule may comprise SEQ ID NO:19 or a functional variant thereof having at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or at least about 99% sequence identity to SEQ ID NO:19. Or, the secondary molecule may comprise SEQ ID NO:19 or a functional variant thereof having at least about 90%, 91%, 92%, 93%, 94% or at least about 95% sequence identity to SEQ ID NO:21.

The term "thrombin" may indicate a thrombin from any species, for example bovine thrombin or human thrombin. The skilled person is readily able to identify alternative suitable thrombin molecules.

The recombinant DNA sequence encoding a fusion protein may be selected from SEQ ID NOs:4-7, or equivalent sequences to any of these in which codons have been altered but wherein the sequence encodes for the same amino acid sequence. The fusion protein may have an amino acid sequence selected from SEQ ID NOs:13-16, or for a functional variant of any of these having at least about 60%, for example at least about 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or at least about 99% sequence identity with the non-variant sequence. A functional variant may comprise SEQ ID NO:19 or 21. Therefore, the recombinant DNA sequence may be any which encodes for any of SEQ ID NO:13-16, or for a functional variant as described. Determination of sequence identity at a global level may be carried out as described above. Other proteins which may be electrostatically modified for use in the invention are listed in Table 4:

Uniprot ID	**Protein**	**Organism**	**SEQ ID NO:**
A8AWJ3_STRGC	CshA	*Streptococcus gordonii*	20
Q54194_STRGN	CshA (variant)	*Streptococcus gordonii*	27
Q8VP45_STRGN	CshB	*Streptococcus gordonii*	28
Q9KWR3_STRGN	Hsa	*Streptococcus gordonii*	29
Q48S75_STRPM	AspA	*Streptococcus pyogenes*	30
Q8E589_STRA3	BspA	*Streptococcus agalactiae*	31
PFBA_STRR6	PfbA	*Streptococcus pneumoniae*	32
Q8GH87_MORCA	UspA1	*Moraxella catarrhalis*	33
Q51227_NEIME	OpcA	*Neisseria meningitidis*	34
Q4U4F4_FUSNU	FadA	*Fusobacterium nucleatum*	35
FIMH_ECOLI	FimH	*Escherichia coli*	36
MRKD_KLEPN	MrkD	*Klebsiella pneumoniae*	37
CSGA_ECOLI	CsgA	*Escherichia coli*	38

Table 4: listing of suitable adhesion proteins

Reference to "CshA", as used throughout this specification, may refer to SEQ ID NO:20 or 27, for example, SEQ ID NO:20.

The recombinant DNA sequence may be expressed according to any routine method, for example, using any expression system such as expression in *E. coli*, in accordance with the routine abilities of the skilled person. Isolation of the expressed anchor protein from the expression system is also within the routine abilities of the skilled person.

A third aspect of the invention provides a method of labelling a cell with a protein label, comprising a method according to the second aspect of the invention, wherein the phospholipid bilayer of step (b) forms the external membrane of the cell and the protein-polymer surfactant conjugate comprises the protein label. The protein label may be the anchor protein or the secondary molecule, where present. The protein label may be GFP, PsmOrange or magnetoferritin, a protein conjugated to labelling molecule or nanoparticle, an enzymatic protein such as a peroxidase or a phosphotriesterase (such as OpdA from *Agrobacterium radiobacter*, or a functional variant or portion thereof) or a protease (such as thrombin), an enzyme precursor (such as prothrombin), an adhesion or "homing" protein such as an antibody, lectin, integrin or adhesion molecule (for example the protein CshA from *Streptococcus gordonii*, or a functional variant or portion thereof comprising the fibronectin binding domain of CshA such as SEQ ID NO:19, or any of the proteins listed in Table 4), a growth factor (such as PlGF-2 or a functional variant or portion thereof comprising PlGF-$2_{(123-144)}$ (SEQ ID NO:21)), or a carrier protein such as a globin, for example, myoglobin. In addition to the options above, the secondary molecule may additionally be selected from a peptide, polypeptide or other molecule, such as a SpyCatcher or SpyTag motif, biotin or streptavidin

The protein label may be a detectable protein label such as a fluorescent protein, or a protein conjugated to a labelling molecule such as a metal particle, a nanoparticle, a fluorescent dye or a fluorescent probe. The label may be one which facilitates separation of a labelled cell from an equivalent cell which does not comprise the protein-polymer surfactant conjugate. The label may be one which is capable of interacting with or binding to a label-binding moiety; for example, there the protein label is an antibody, the label-binding moiety may be an antigen capable of binding to the antibody; where the protein label is a SpyCatcher polypeptide the label-binding moiety may be a SpyTag polypeptide; where the protein label is a SpyTag polypeptide the label-binding moiety may be a SpyCatcher polypeptide; where the protein label is streptavidin, the label-binding moiety may be biotin. The label-binding moiety itself may be further attached to a cell or form part of a larger construct, molecule or structure, by the methods described herein or by other methods known in the art.

A related aspect of the invention therefore provides a cell obtained by the method according to the third aspect of the invention. Such a cell is typically a phospholipid composition according to the first aspect of the invention.

The term "cell" as used throughout this specification, in relation to any aspect or embodiment of the invention described herein, may be a prokaryotic or eukaryotic cell, for example a bacterial, fungal, protist, plant, insect, reptile, bird, fish or mammal cell, for example a human, dog, cat or horse cell, or a bovine, porcine or ovine cell. The cell may be one which is not a human cell, and/or not a human embryonic cell or cell derived therefrom, and/or not a human embryonic stem cell or cell derived therefrom.

A fourth aspect of the invention provides a method for forming a tissue engineering scaffold, comprising use of a phospholipid composition (such as a cell) according to the first aspect of the invention (for example obtained by the method according to the second or third aspects of the invention), wherein the protein-polymer surfactant conjugate comprises a protein (which may be the anchor protein or may be a secondary molecule) which is, in its naturally occurring form, known to promote growth and/or healing of tissue. For example, the anchor protein may be cationised or supercharged thrombin or prothrombin or may be a cationised or supercharged functional variant or portion of thrombin or prothrombin, or the secondary molecule may be thrombin or prothrombin (e.g., SEQ ID NO:25 or 26) or a functional variant or portion of thrombin or prothrombin, or PlGF-2 (SEQ ID NO:22), or a functional variant or portion thereof comprising SEQ ID NO:21. For example, the method may comprise contacting a cell, which is a phospholipid composition according to the first aspect of the invention wherein the anchor protein is cationised or supercharged thrombin or wherein the secondary molecule is thrombin, with a fibrinogen composition such as a fibrinogen-containing gel, or with a fibrinogen-containing structure formed by the method described in Armstrong *et al*. (*Adv. Healthcare Mat*. (2016) vol. 5 p 1724-1730) and co-pending application PCT/GB2016/053358 (published as WO2017/187114). Alternatively or additionally, the method may comprise contacting a cell, which is a phospholipid composition according to the first aspect of the invention wherein the protein-polymer surfactant conjugate comprises a secondary molecule which is PIGF-2 (SEQ ID NO:22), or a functional variant or portion thereof comprising SEQ ID NO:21, with a material which comprises one or more of fibronectin, vitronectin, tenascine C, osteopontin and/or fibrinogen. The material may be an extracellular matrix and/or gel and/or or a structure formed by the method described in Armstrong *et al*. (*Adv. Healthcare Mat*. (2016) vol. 5 p 1724-1730) and co-pending application PCT/GB2016/053358 (published as WO2017/187114).

A fifth aspect of the invention provides a tissue engineering scaffold comprising a phospholipid composition (such as a cell) according to the first aspect of the invention, for example a scaffold prepared by the method according to the fourth aspect of the invention. The protein-polymer surfactant conjugate may comprise a protein (which may be the anchor protein or may be a secondary molecule) which is, in its naturally occurring anchor precursor protein form, known to promote growth and/or healing of tissue. For example, the

anchor protein may be cationised or supercharged thrombin or prothrombin or a cationised or supercharged functional variant or portion of thrombin or prothrombin, or the secondary molecule may be thrombin or prothrombin (e.g., SEQ ID NO:25 or 26) or a functional variant or portion of thrombin or prothrombin, or may be PlGF-2 (SEQ ID NO:22) or a functional variant or portion thereof comprising SEQ ID NO:21. The tissue engineering scaffold may be formed by a method comprising the method described in Armstrong *et al*. (*Adv. Healthcare Mat*. (2016) vol. 5 p 1724-1730) and co-pending application PCT/GB2016/053358 (published as WO2017/187114). Alternatively or additionally, the tissue engineering scaffold may comprise one or more of fibronectin, vitronectin, tenascine C, osteopontin and/or fibrinogen.

A sixth aspect of the invention provides a method of promoting tissue growth and/or healing, comprising use of a cell which is (or comprises) a phospholipid composition according to the first aspect of the invention (for example obtained by the method according to the second aspect of the invention), wherein the protein-polymer surfactant conjugate comprises a protein (which may be the anchor protein or may be a secondary molecule) which is, in its naturally occurring anchor precursor protein form, known to promote growth and/or healing of the tissue, by introducing the cell to a site where the tissue is desired to grow and/or heal. For example, the cell may be a mesenchymal stem cell and the anchor protein may be cationised or supercharged thrombin or prothrombin (e.g., SEQ ID NO:25 or 26) or a cationised or supercharged functional variant of thrombin or prothrombin, or the secondary molecule may be thrombin or prothrombin (e.g., SEQ ID NO:25 or 26) or a functional variant or portion of thrombin or prothrombin, or PlGF-2 (SEQ ID NO:22), or a functional variant or portion thereof comprising SEQ ID NO:21. The tissue may be *in vitro* or *ex vivo*, or may be *in vivo* within an animal, for example a mammal such as a human, dog, cat or horse. The method may comprise use of a scaffold according to the fifth aspect of the invention, and/or of a pharmaceutical composition according to the eleventh aspect of the invention, and/or of a surgical composition according to the twelfth aspect of the invention.

A seventh aspect of the invention provides a method of targeting a cell to a tissue, comprising use of a cell which is a phospholipid composition according to the first aspect of the invention (for example obtained by the method according to the second aspect of the invention), wherein the phospholipid bilayer forms at least a portion of the external membrane of the cell and the protein-polymer surfactant conjugate comprises a protein (which may be the anchor protein or a secondary molecule) specific for the tissue, such as an antibody, lectin, integrin or adhesin. The tissue may be *in vitro* or *ex vivo*, or may be *in vivo* within an animal, for example a mammal such as a human, dog, cat or horse. The tissue may be cardiac tissue and the cell may be a mesenchymal stem cell or a cardiomyocyte, in which case the protein-polymer surfactant conjugate may comprise a secondary molecule which is CshA or a functional variant or portion thereof comprising SEQ

ID NO:19. The method may comprise introducing the cell to a system (which may be a system which is not a human or animal body) in which the tissue is present, such as a tissue culture container, an *ex vivo* tissue (for example, one obtained from an individual suffering from myocardial infarction, cardiomyopathy and/or myocarditis), or a body comprising the tissue, for example a body suffering from myocardial infarction, cardiomyopathy and/or myocarditis. The tissue may, therefore, be *in vitro* or *ex vivo*, or may be *in vivo* within an animal, for example a mammal such as a human, dog, cat or horse. The method may comprise use of a scaffold according to the fifth aspect of the invention, and/or of a pharmaceutical composition according to the eleventh aspect of the invention, and/or of a surgical composition according to the twelfth aspect of the invention.

An eighth aspect of the invention provides a method of delivering a protein to the interior of a cell, comprising the method according to the second aspect of the invention, wherein the phospholipid bilayer forms the external membrane of the cell. This is enabled by allowing or promoting the process of endocytosis, well known to the skilled person, by which portions of the cell membrane and/or molecules associated with the membrane are internalised into the cell. The composition of the protein-polymer surfactant conjugate may promote or inhibit the process, i.e., promoting or inhibiting the speed/rate of endocytosis of the embedded protein-polymer surfactant conjugate. The cell may be a cell which is not a human embryonic cell or human embryonic stem cell. The cell may be a cell which is *in vitro* or *ex vivo*, i.e. a cell which is not *in vitro* within a human or animal body.

A ninth aspect of the invention provides a phospholipid composition according to the first aspect of the invention, for use in therapy. The phospholipid composition may be a cell such as a mesenchymal stem cell. In the ninth aspect, the phospholipid composition may be for use in a method of promoting tissue growth and/or healing, wherein the embedded protein-polymer surfactant conjugate comprises a protein (which may be the anchor protein or a secondary molecule) known to promote growth and/or healing of the tissue. For example, the protein may be cationised or supercharged thrombin or prothrombin or a cationised or supercharged functional variant of thrombin or prothrombin, or the protein-polymer surfactant conjugate may comprise a secondary molecule which is thrombin or prothrombin (e.g., SEQ ID NO:25 or 26) or a functional variant or portion of thrombin or prothrombin, or PlGF-2 (SEQ ID NO:22), or a functional variant or portion thereof comprising SEQ ID NO:21. Alternatively, the protein known to promote growth and/or healing of the tissue may be myoglobin, in which case the tissue may be cartilage. Alternatively, the secondary molecule may be CshA or a functional variant or portion thereof comprising SEQ ID NO:19, in which case the tissue may be heart tissue and the composition may be for use in a method for the treatment of myocardial infarction, cardiomyopathy and/or myocarditis. In this case, the cell may be a cardiomyocyte. The tissue may be *in vitro* or *ex vivo*, or may be *in vivo* within an animal, for example a mammal such as a human, dog, cat or horse. The method may

comprise use of a scaffold according to the fifth aspect of the invention, and/or of a pharmaceutical composition according to the eleventh aspect of the invention and/or of a surgical composition according to the twelfth aspect of the invention.

Alternatively, in the ninth aspect of the invention the phospholipid composition according to the first aspect of the invention may be for use in the treatment of a poisoned human or animal, for example a human or animal which has been contacted with a poison. A tenth aspect of the invention provides a method for the treatment of a poisoned human or animal, for example a human or animal which has been contacted with a poison, comprising contacting the human or animal with a composition according to the first aspect of the invention, or administering such a composition to the human or animal in a therapeutically effective amount. "Contacted with a poison" may indicate that the human or animal has ingested the poison with or as food or drink, or inhaled the poison, or absorbed the poison after contact with the skin or other exterior body surface, or internalised the poison by any other means. The term "poisoned" is a routinely used term which may indicate that the normal health of the human or animal is reduced or negatively impacted following the contact with the poison and the term "treatment" may indicate that the health of the human or animal is improved or restored to a pre-poisoning state. "Treatment" may encompass avoidance or prevention of death of the human or animal where this would have been the expected outcome after contact of the human or animal with the poison, in the absence of treatment in accordance with the ninth aspect of the invention. Reduced and/or improved health may be determined by any routine measure, for example, occurrence or reduction of a rash, bleeding, vomiting, diarrhoea, increased temperature, dehydration, weight loss, sight loss, hearing loss, muscle spasm and/or paralysis, by way of non-limiting example. The term "poison" encompasses any substance which adversely impacts the normal functioning of a cell or organism, including a human or animal body or a plant, and includes toxins and venoms, as well as a pesticide or a nerve agent, by way of non-limiting example.

In the ninth or tenth aspect of the invention, the composition according to the first aspect of the invention may (a) comprise an anchor protein which is an enzyme which can neutralise the poison; or (b) comprise an anchor protein which is linked to a secondary molecule which can bind to or neutralise the poison. The term "neutralise" indicates that the poison substance is broken down or otherwise altered (or its effect in the human or animal body is altered) so that it is no longer toxic (i.e., harmful to the human or animal), or so that the toxicity is reduced. The anchor protein may be cationised or supercharged OpdA, or a cationised or supercharged functional variant or portion thereof capable of degrading an organophosphorus compound, or the secondary molecule may comprise OpdA or a functional variant or portion thereof capable of degrading an organophosphorus compound.

In the sixth, seventh, eighth, ninth or tenth aspects of the invention, the phospholipid composition may be in the form of a pharmaceutical composition, which forms an eleventh aspect of the invention, further comprising a pharmaceutically acceptable carrier, diluent or vehicle. For example, the pharmaceutical composition may be in the form of a sterile injectable preparation which may be an aqueous or an oleaginous suspension, or a suspension in a non-toxic parenterally-acceptable diluent or solvent. The aqueous suspension may be prepared in, for example, mannitol, water, Ringer's solution or isotonic sodium chloride solution. Alternatively, it may be prepared in phosphate buffered saline solution. The oleaginous suspension may be prepared in a synthetic monoglyceride, a synthetic diglyceride, a fatty acid or a natural pharmaceutically-acceptable oil. The fatty acid may be an oleic acid or an oleic acid glyceride derivative. The natural pharmaceutically-acceptable oil may be an olive oil, a castor oil, or a polyoxyethylated olive oil or castor oil. The oleaginous suspension may contain a long-chain alcohol diluent or dispersant, for example, conforming to Ph. Eur. and/or Ph. Helv. The pharmaceutical composition may comprise one or more pharmaceutically or otherwise biologically active agents in addition to the phospholipid composition of the invention. For example, the composition may include a therapeutic agent such as a conventional drug, antibody or other protein component.

A twelfth aspect of the invention provides a surgical composition comprising the phospholipid composition according to the first aspect of the invention, and fibrinogen and/or a surgically acceptable carrier, diluent or vehicle, for example any those mentioned above for the eleventh aspect of the invention. A surgically acceptable carrier, diluent or vehicle may comprise a hydrogel. The surgically acceptable carrier may be a scaffold material such as a membrane or a fabric. The scaffold material may be formed by a method comprising the method described in Armstrong *et al*. (*Adv. Healthcare Mat*. (2016) vol. 5 p 1724-1730) and co-pending application PCT/GB2016/053358 (published as WO2017/187114). In some embodiments, the surgical composition may be referred to herein as a "surgical glue".

A thirteenth aspect of the invention provides a method of decontaminating a sample comprising a poison, comprising either: (a) contacting the sample with a composition according to the first aspect of the invention, wherein the anchor protein is an enzyme which can neutralise the poison; or (b) contacting the sample with a composition according to the first aspect of the invention, wherein the anchor protein is linked to a secondary molecule which can bind to or neutralise the poison. The term "neutralise" indicates that the poison substance is broken down or otherwise altered so that it is no longer toxic, or so that the toxicity is reduced. A sample may be any liquid sample (such as a water sample) or a solid sample (which may be any solid surface, or land, or a soil sample, or fabric, for example clothing or material for use as bed linen). The poison may be any chemical which is toxic to humans, animals, insects, fish and/or plants, for example a pesticide or a nerve

agent. In the ninth aspect of the invention, the anchor protein, or the secondary molecule when present, may be an organophosphate hydrolase enzyme such as OpdA obtained from *Rhizobium radiobacter*, or a homologous enzyme obtained from another organism or adapted from such an enzyme.

A fourteenth aspect of the invention provides a polypeptide comprising a fusion protein of: a supercharged anchor protein and any of SEQ ID NOs:19-38; or a supercharged anchor protein and a functional variant of any of any of SEQ ID NOs:19-38 having at least 60% sequence identity thereto. For example, the supercharged anchor protein may be scGFP and the polypeptide may comprise at least one of SEQ ID NOs:13-16 or a functional variant of any of these having at least about 60%, for example at least about 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or at least about 99% sequence identity with the non-variant sequence. In an alternative fourteenth aspect of the invention, there is provided a polypeptide comprising a supercharged version of SEQ ID NO:10, also described by SEQ ID NO:39 (e.g., comprising SEQ ID NO:11, also described by SEQ ID NO:40), or comprising a supercharged version of any of SEQ ID NOs:10, 19-22 or 25-39. A fifteenth aspect of the invention provides a recombinant nucleic acid sequence (i.e., a polynucleotide), such as a DNA sequence, encoding for a polypeptide according to the fourteenth aspect of the invention, for example, comprising at least one of SEQ ID NOs:2 or 4-7, or equivalent sequences to any of these in which codons have been altered but wherein the sequence encodes for the same amino acid sequence. The polypeptide according to the fourteenth aspect of the invention or the nucleic acid according to the fifteenth aspect of the invention may be for use in therapy; for example, where the polypeptide comprises any of SEQ ID NO:14, 19, 20 or 27-38, the polypeptide according to the fourteenth aspect of the invention or the nucleic acid according to the fifteenth aspect of the invention may be for use in a method of treatment of myocardial infarction, cardiomyopathy and/or myocarditis.

Therefore, a sixteenth aspect of the invention provides a therapeutic method comprising administering a polypeptide according to the fourteenth aspect of the invention or the nucleic acid according to the fifteenth aspect of the invention, or a pharmaceutical composition comprising such a polypeptide or nucleic acid sequence, to a subject requiring therapeutic treatment. Such a pharmaceutical composition may by in a form as described above in relation to the eleventh aspect of the invention. For example, the pharmaceutical composition may further comprise a pharmaceutically acceptable carrier, diluent or vehicle, as described above. The method may be a method of treatment of myocardial infarction, cardiomyopathy and/or myocarditis comprising administering a therapeutic amount of a polypeptide comprising any of SEQ ID NO:14, 19, 20 or 27-38, or a nucleic acid sequence encoding for one or more of these, or a pharmaceutical composition comprising one or more such polypeptide or nucleic acid sequence, to a subject in need thereof (i.e., a subject suffering from one or more of myocardial infarction, cardiomyopathy and/or myocarditis).

Throughout the description and claims of this specification, the words "comprise" and "contain" and variations of the words, for example "comprising" and "comprises", mean "including but not limited to", and do not exclude other components, integers or steps. Throughout the description and claims of this specification, the singular encompasses the plural unless the context otherwise requires. In particular, where the indefinite article is used, the specification is to be understood as contemplating plurality as well as singularity, unless the context requires otherwise.

Other features of the present invention will become apparent from the following examples. Generally speaking, the invention extends to any novel one, or any novel combination, of the features disclosed in this specification (including the accompanying claims and drawings). Thus, features, integers, characteristics, compounds or chemical moieties described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any other aspect, embodiment or example described herein, unless incompatible therewith.

Moreover, unless stated otherwise, any feature disclosed herein may be replaced by an alternative feature serving the same or a similar purpose.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described, by way of example only, with reference to the accompanying drawings, in which:

Figure 1 shows the reaction pathway for the covalent alteration of glutamic acid and aspartic acid residues via a nucleophilic addition-elimination mechanism;

Figure 2 is a diagram showing a protein-polymer surfactant conjugate (or "protein-surfactant bioconjugate") inserted into a phospholipid bilayer;

Figure 3 is a diagram showing a protein-polymer surfactant conjugate comprising an anchor protein and a secondary molecule, with the secondary molecule positioned outside the membrane;

Figure 4 shows the chemical structure of various surfactants used in the work described herein (for S621 and S907 x = 11-13, for S621 y = 7-9, for S907 y = 14-15);

Figure 5 shows the mass spectrum of scGFP-OpdA;

Figure 6 shows (a) the UV-vis absorbance of [scGFP–CshA] and [scGFP–CshA][S] and (b) the fluorescence excitation and emission of [scGFP–CshA] and [scGFP–CshA][S];

Figure 7 shows UV–vis absorbance of scGFP-OpdA;

Figure 8 shows excitation and emission properties of scGFP-OpdA;

Figure 9 shows mass spectra of thrombin and cationised thrombin at a charge number of 3;

Figure 10 shows the zeta potentiometries of OpdA, cOpdA and [cOpdA][S];

Figure 11 shows the change in zeta potential over time during cationisation of thrombin;

Figure 12 shows (a) the secondary structure composition of OpdA, cOpdA, and [cOpdA][S], and (b) the thermal denaturation of OpdA, cOpdA, and [cOpdA][S];

Figure 13 shows the radial probability distribution of OpdA, cOpdA, and [cOpdA][S];

Figure 14 shows (a) the circular dichroism of scGFP–CshA and (b) [scGFP–CshA][S] at 30, 60 and 90°C;

Figure 15 shows the number of scGFP–CshA and [scGFP–CshA][S] molecules bound per hMSC;

Figure 16 shows the fluorescence of the primed hMSCs;

Figure 17 shows the cytotoxicity of scGFP–CshA and [scGFP–CshA][S];

Figure 18 shows the proliferation of hMSCs primed with [scGFP_CshA] and [scGFP_CshA][S];

Figure 19 shows the Michaelis–Menten parameters of OpdA, cOpdA, and [cOpdA][S];

Figure 20 shows the change in enzymatic activity over time during cationisation of thrombin;

Figure 21 shows a confocal micrograph of [Thrombin][S]-catalysed fibrin gel;

Figure 22 shows the cell adherence of unlabelled hMSCs, scGFP–CshA primed hMSCs and [scGFP–CshA][S] primed hMSCs on BSA-coated and Fn-coated plates; and

Figure 23 shows scanning electron microscopy images of explanted bovine articular cartilage with hMSCs and scGFP–PlGF$_{(123–144)}$-primed hMSCs.

Examples

General methods

Plasmid preparation

The OpdA gene was acquired in a pETMCSI vector, and required no further processing. The scOpdA gene was inserted into a pETMCSI vector via Gibson assembly, as described previously in Gibson et al. (Nat Methods. (2009) April 12;6(5):343-5.). scGFP–CshA, scGFP–SpyCatcher, mCherry–SpyTag, CshA–SpyTag, scGFP–OpdA, and scGFP–PlGF genes

were inserted into pOPINF vectors via the In-Fusion™ cloning system, according to manufacturer's instructions. The vectors were amplified via transformation into Stellar cells (Clontech, US) or Top10 cells (Thermo Fisher Scientific, US), followed by miniprep purification (Qiagen, Germany), each according to the manufacturer's instructions. DNA and amino acid sequences are listed in Table 5:

Protein/construct as referred to herein	Gene sequence	Amino acid sequence
OpdA	SEQ ID NO:1	SEQ ID NO:10; SEQ ID NO:39
scOpdA	SEQ ID NO:2	SEQ ID NO:11; SEQ ID NO:40
scGFP	SEQ ID NO:3	SEQ ID NO:12
scGFP–OpdA	SEQ ID NO:4	SEQ ID NO:13
scGFP–CshA	SEQ ID NO:5	SEQ ID NO:14
scGFP–PlGF$_{(123-144)}$	SEQ ID NO:6	SEQ ID NO:15
scGFP–SpyCatcher	SEQ ID NO:7	SEQ ID NO:16
mCherry–SpyTag	SEQ ID NO:8	SEQ ID NO:17
CshA–SpyTag	SEQ ID NO:9	SEQ ID NO:18
Fibronectin binding domain of CshA	-	SEQ ID NO:19
CshA	-	SEQ ID NO:20
PlGF-2$_{(123-144)}$	-	SEQ ID NO:21
PlGF-2	-	SEQ ID NO:22
SpyCatcher	-	SEQ ID NO:23
SpyTag	-	SEQ ID NO:24
bovine prothrombin	-	SEQ ID NO:25
human prothrombin	-	SEQ ID NO:26

Table 5: Protein gene and amino acid sequences utilised herein

Protein expression

OpdA, scOpdA, scGFP–CshA, scGFP–OpdA, and scGFP–PlGF$_{(123-144)}$ were obtained by expression in BL21(DE3) cells (New England Biolabs, USA), transformed with vectors containing their respective genes, using routine methods. Protein specific parameters are outlined in Table 6. Bovine thrombin and human fibrinogen were obtained from commercial sources (Sigma, Cat. No T7326 and F3879, respectively).

Protein	Medium	Temperature	Induction
OpdA	Terrific broth, with 100 µM $CoCl_2$	30°C	None
scOpdA	Terrific broth, with 100 µM $CoCl_2$	30°C	1 mM IPTG when $Abs_{600} \geq 0.6$
scGFP–OpdA	Terrific broth, with 100 µM $CoCl_2$, 10 g/L NaCl	37°C	1 mM IPTG when $Abs_{600} \geq 0.6$
scGFP–CshA	Terrific broth	37°C	1 mM IPTG when $Abs_{600} \geq 0.6$
scGFP–PlGF$_{(123-144)}$	Lysogeny broth	37°C	1 mM IPTG when $Abs_{600} \geq 0.6$
scGFP–SpyCatcher	Terrific broth	37°C	1 mM IPTG when $Abs_{600} \geq 0.6$
mCherry–SpyTag	Terrific broth	37°C	1 mM IPTG when $Abs_{600} \geq 0.6$
CshA–SpyTag	Terrific broth	37°C	1 mM IPTG when $Abs_{600} \geq 0.6$

Table 6: Protein expression parameters

Protein purification

Lysis buffer was added to cell pellets and lysed using pulse sonication, using routine methods. The protein was then purified using fast protein liquid chromatography (FPLC). Proteins were further purified using size exclusion chromatography, using routine methods. Protein specific purification steps are outlined in Table 7. No purification was required for the commercially purchased thrombin or fibrinogen.

Protein	Method	Lysis buffer	Elution buffer
OpdA	Anion exchange (DEAE column)	30 mM HEPES, 100 μM $CoCl_2$, pH 8	N/A
scOpdA	IMAC (Ni–NTA column)	30 mM HEPES, 1.5 M NaCl, 20 mM imidazole, pH 8	30 mM HEPES, 1.5 M NaCl, 1 M imidazole, pH 8
scGFP–OpdA	IMAC (Ni–NTA column)	20 mM Sodium phosphate, 1 M NaCl, 2 nM $MgCl_2$, 50 mM imidazole, pH 8	20 mM Sodium phosphate, 1 M NaCl, 2 nM $MgCl_2$, 500 mM imidazole, pH 8
scGFP–CshA	IMAC (Ni–NTA column)	20 mM Tris–HCl, 1 M NaCl, 20 mM imidazole, pH 7.5	20 mM Tris–HCl, 1 M NaCl, 500 mM imidazole, pH 7.5
scGFP–PlGF$_{(123-144)}$	IMAC (Ni–NTA column)	20 mM Tris–HCl, 1 M NaCl, 20 mM imidazole, pH 7.5	20 mM Tris–HCl, 1 M NaCl, 500 mM imidazole, pH 7.5
scGFP–SpyCatcher	IMAC (Ni–NTA column)	20 mM Tris–HCl, 1 M NaCl, 20 mM imidazole, pH 7.5	20 mM Tris–HCl, 1 M NaCl, 500 mM imidazole, pH 7.5
mCherry–SpyTag	IMAC (Ni–NTA column)	20 mM Tris–HCl, 1 M NaCl, 20 mM imidazole, pH 7.5	20 mM Tris–HCl, 1 M NaCl, 500 mM imidazole, pH 7.5
CshA–SpyTag	IMAC (Ni–NTA column)	20 mM Tris–HCl, 1 M NaCl, 20 mM imidazole, pH 7.5	20 mM Tris–HCl, 1 M NaCl, 500 mM imidazole, pH 7.5

Table 7: Protein purification conditions

Synthesis of glycolic acid ethoxylate 4-nonylphenyl ether (oxidised IGEPAL-CO890) surfactant

Surfactant was prepared as described in Armstrong *et al*. (Nat. Commun. (2015) Jun 17;6:7405). Briefly, 2 g IGEPAL CO-890 dissolved in 50 mL deionised-water was mixed with 30 mg 2,2,6,6,-tetramethyl-1-piperidinyloxyl (TEMPO), 50 mg NaBr, and 5 mL NaClO solution containing 10-15% available chlorine. The solution was periodically adjusted to pH 11 and stirred for 24 hours. The reaction was quenched with ethanol and adjusted to pH 1. Solvent extraction was performed with 3 washes of 80 mL aliquots of chloroform, then 3 washes with 80 mL aliquots of deionised water adjusted to pH 1. The resulting solution was dried under reduced pressure at 40°C. The remaining solid was redissolved in 40 mL ethanol, recrystallised at -20°C, the ethanol decanted, and the crystals dried under reduced pressure at 65°C.

Protein–surfactant conjugation

To form the conjugated constructs, glycolic acid ethoxylate 4-nonylphenyl ether was added to a solution of cationised protein or protein comprising supercharged GFP or OpdA (see below). Any excess surfactant may be removed via dialysis, using 14,000 MWCO tubing. The specific parameters are presented in Table 8.

Protein	**Surfactant form**	**Moles of surfactant per cationic site**	**Buffer**	**Time**	**Temperature**
cationised OpdA	10 mg/mL solution	1	30 mM HEPES, 100 μM $CoCl_2$, pH 8	1 hour	4°C
cationised Thrombin	Solid	1.4	60 mM HEPES, pH 7	1 hour	Room temperature
scGFP–CshA/ OpdA/ $PlGF_{(123-144)}$	25 mg/mL solution	1.4	20 mM Tris–HCl, pH 7.5	Overnight	4°C

Table 8: Conjugation parameters

Mass spectrometry

Mass spectrometry was performed using a Bruker ultrafleXtreme MALDI–TOF/TOF mass spectrometer in linear positive mode. The matrix was a saturated solution of either sinapinic acid or α-hydroxycinnamic acid in a mixture of equal volumes acetonitrile and water, with a final concentration of 0.1% trifluoroacetic acid. 0.5 μL of 1:1 sample and matrix mixture was spotted on a ground steel plate for analysis.

Dynamic light scattering and zeta potentiometry

Dynamic light scattering (DLS) and zeta potentiometry analyses were performed on a Zetasizer Nano SP (Malvern Instruments, UK), and the data analysed using Zetasizer software (Malvern Instruments).

Small angle X-ray scattering

Small angle X-ray scattering was performed on the B21 beamline at the Diamond Light Source, Oxford. Samples were concentrated with 10,000 MWCO spin concentrators and flow-through retained for use as backgrounds. The samples were then spun through 1,000,000 MWCO spin concentrators to remove large contaminants. Samples were exposed for 18 frames of 10 seconds each. Data analyses were performed with the ScÅtter software package, using ATSAS plugins.

Cell culture

Human mesenchymal stem cells (hMSCs) were harvested from the proximal femur bone marrow of osteoarthritic patients undergoing total hip replacement surgery, in full accordance with Bristol Southmead Hospital Research Ethics Committee guidelines (reference #078/01), and having received informed consent from all patients. Cells were cultured at 5% CO2, using low-glucose DMEM, supplemented with 10% fetal bovine serum, 2 mM GlutaMAX (Gibco, US), 100 µg/mL penicillin-streptomycin and 5 ng/mL freshly supplemented basic human Fibroblast Growth Factors (FGF) (Peprotech, USA).

Cell priming

Cells were washed with PBS, and suspended with trypsin–EDTA solution (Sigma, UK). The protein solution was added to the suspended cells in phenol free DMEM, and left to shake and incubate at 37°C for 15 minutes. The cells were then centrifuged at 500 g for 5 minutes, and the supernatant discarded. The cells were then resuspended for immediate use or to be plated.

Alternatively, a protein solution was applied directly to plated cells. The cells were washed with PBS, and the protein solution added in an appropriate buffer for up to 30 minutes with shaking at 37°C. The cells were then washed with PBS again, and ready for use.

Cell cytotoxicity assays

The cytotoxicity of the constructs was assayed using either (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) or alamarBlue® according to the manufacturers' instructions. Briefly, hMSCs were plated in 96-well plates at a range of concentrations to produce a standard curve. A known quantity of cells was primed with a solution of the construct for 15 minutes then washed with PBS before incubation with either MTS or alamarBlue solution for 1–2 hours. Absorbance or

fluorescence values were then collected using a plate reader, and the values compared against the standard curve to determine the percentage survival of primed cells.

UV–visible and fluorescence spectrophotometry

UV–visible and fluorescence spectrophotometry were performed using routine methods.

Bicinchoninic acid assay

Bicinchoninic acid assays were performed according to the manufacturer's instructions. Briefly, 20 µL of samples were added to 200 µL of reagents A and B (Thermo Scientific, UK) mixed in a 50:1 ratio in a 96-well plate. The plate was then incubated for 30 minutes at 37°C, before measuring the absorbance at 530 nm using a plate reader. Absorbance values collected for analytes were compared against a standard curve of a protein at known concentrations to calculate the concentration of the analytes.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE analysis was performed using routine methods. Briefly, analytes were mixed 1:1 with sample application buffer comprising glycerol, SDS, EDTA, Tris, mercaptoethanol and bromophenol blue, and heated to 95°C for 5 minutes. The samples were then loaded into Novex® 4–20% Tris–glycine pre-cast gels (Thermo Fisher Scientific). A voltage of 200 V was applied for 50 minutes, and the resultant gel stained with Coomassie Blue stain.

Circular dichroism

Synchrotron-radiation circular dichroism was performed on the B23 beamline at the Diamond Light Source, Oxford. Samples were desalted into chloride-free buffers. Spectra were collected from 185 to 260 nm, using a cuvette with a pathlength of 200 µm. For thermal studies, data were collected from 20 to 90 to 20°C at 5°C intervals with 1 minute incubation time. Alternatively, lamp-radiation circular dichroism was performed on a J-1500 CD spectrophotometer (JASCO, Germany), using a 100 µm pathlength cuvette. Data deconvolution was performed using the BeStSel web service. (Micsonai et al. (2015) Proc. Natl. Acad. Sci. U.S.A. 112, E3095-3103).

Fluorescence microscopy

Confocal microscopy was performed using a Leica TCS SP8 confocal laser scanning fluorescence microscope (Leica Microsystems, Germany), using routine methods. Widefield microscopy was performed using a Leica DMI6000 inverted epifluorescence microscope (Leica Microsystems, Germany), using routine methods. OpdA and thrombin were fluorescently tagged with either 5(6)–carboxyfluorescein N-hydroxysuccinimide (Sigma) or rhodamine N-hydroxysuccinimide (Thermo Scientific, Germany), according to the manufacturer's instructions, whereas scGFP-based constructs are inherently fluorescent. To observe localization of the complexes, proteins were added to cells plated in a glass-bottom dish for 10–30 minutes, washed with PBS, then imaged.

Scanning Electron Microscopy

Bovine articular surface samples were fixed with 2.5% glutaraldehyde for 1 hour, rinsed three times for 10 minutes with 100 mM sodium phosphate buffer pH 7.4, placed in 1% osmium tetroxide for one hour, washed three times for 10 minutes with 100 mM sodium phosphate buffer, then washed with water for 10 minutes. Dehydration steps were made with 25, 50, 70, 80, 90, 96, and 100% ethanol, changing concentration every 10 minutes, and then processed with a critical point dryer. The samples were sputter coated with palladium or chromium and imaged on an FEI field emission scanning electron microscope (Quanta 200).

Proliferation

Proliferation of tissue engineered hMSCs within [cThrombin][S] catalysed fibrin constructs was analysed by comparing the results of MTS assays (described above in 'Cell cytotoxicity assays') performed over time. The effect of priming hMSCs with [scGFP–CshA] and [scGFP-CshA][S] on their proliferation was analysed using a haemocytometer to count cells, and comparing them to their seeding number.

Flow cytometry

hMSCs primed with protein complexes were harvested, washed in an initial wash step, and centrifuged at 1500 RPM for five minutes. The sediment was re-suspended in PBS containing a dead stain. Suspensions containing approximately 1,000,000 cells per mL were transferred to individual flow cytometry tubes, and analysed using a flow cytometer and associated software. The cell suspension was passed through the interrogation point at a rate of 100-300 events per second with a total of 20,000 whole cell events measured. The side scatter area (SSC-A), forward scatter area (FSC-A), forward scatter height (FSC-H), and experiment-specific fluorescence were measured, with unlabelled cells as a control group to define the gated areas used for all samples. The whole cell populations were defined by an FSC-A vs SSC-A gate firstly, with data outside this region excluded as cell debris. Following this, the whole cell populations were gated by FSC-A vs FSC-H defining the single cell populations. The single cell populations were further gated by defining an upper limit on the FSC-A vs the dead stain filter dot plot, and data above this limit were excluded as dead cells. The live cells were gated on a FSC-A vs. FITC-A plot, with data inside the region corresponding to scGFP positive labelled cells and data outside the region corresponding to non-fluorescent cells (priming hMSCs with scGFP–CshA constructs), or were gated on a PE-CF594-A vs. FITC-A plot, with data inside the region corresponding to scGFP positive (Q1 and Q2) and mCherry positive labelled cells (Q2 and Q4), and data outside the region corresponding to non-fluorescent cells (Q3) (cell-surface scGFP–SpyCatcher and mCherry SpyTag reaction). Experiment-specific parameters are given in Table 9.

Experiment	Initial wash step	Dead staining	Fixing solution	Instrument and software	Filters for measuring fluorescence
scGFP–CshA priming	Phenol-free DMEM	0.004 mg/mL Propidium iodide in PBS	No fixing	NovoCyte, NovoExpress	Qdot 605-A (propidium iodide), FITC-A (scGFP)
scGFP–SpyCatcher and mCherry–SpyTag cell-surface reaction	Phenol-free DMEM then PBS	1%(v/v) Zombie NIR in PBS for 15 minutes at room temperature, then washed with PBS and fixed	1% para-formaldehyde	LSR Fortessa X20, FACSDiva	APC-Cy7-A (Zombie NIR), FITC-A (scGFP), PE-CF594-A (mCherry)

Table 9: Flow cytometry parameters

Cell membrane uptake quantification

hMSCs were primed for 15 minutes using protein (e.g. [scGFP–CshA]) and conjugate (e.g. [scGFP–CshA][S]) at a range of concentrations in phenol-free DMEM. The amount of protein bound to cell membranes could be calculated by subtracting the amount of protein in the supernatant, determined using UV–visible spectrophotometry at 487 nm, from the amount of protein added to the cells.

Sedimentation velocity analytical ultracentrifugation (SV-AUC)

SV-AUC experiments were performed on a Beckman Optima XL-I (Beckman Coulter, USA) using the UV/Visible absorption system at 280 nm and 487 nm, at 40,000 rpm and at 20° C using two channel 12 mm Epon centerpieces. Buffer density and viscosity was determined using a Lovis 2000 rolling ball viscometer (Anton Paar, Austria). Sedimentation coefficients (S) were determined using the continuous distribution Lamm equation model (c(S)) and were converted to standard conditions (Sw (20, w)). Molecular weights were calculated directly from integrated c(s) peaks.

Chemical cationisation methods

Protein cationisation

Protein (OpdA pr thrombin) was cationised using a method derived from that described in Armstrong et al. (Nat. Commun. (2015) Jun 17;6:7405). Briefly, a solution of protein (OpdA or thrombin) in HEPES buffer was added to pH-neutralised N-N′-dimethyl-1,3-propanediamine (DMPA) at a given ratio, and the solution pH-adjusted with 6M HCl. N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) was added either in a single addition or two half additions, and the solution was left to stir, then desalted using

buffer exchange with 10K MWCO spin concentrators to end the reaction. Specific experimental parameters for each protein are presented in Table10. Performing the method as reported by Armstrong *et al.* would lead to severe loss of enzyme activity, as the cationisation reaction leads to inactivation over time. The inventors have determined that either limiting the reaction time or performing size exclusion chromatography on the crude cationisation solution produces active enzyme.

Protein	**Buffer**	**Ration DMPA : anionic sites**	**Ratio EDC : anionic sites**	**Reaction pH**	**Reaction time**	**Reaction temp.**	**Purification**
OpdA	30 mM HEPES, 100 µM $CoCl_2$	300:1	50:1	5.1	24 hours	4°C	Size exclusion chromatography
Thrombin	60 mM HEPES	150:1	34:1	6.5	1 hour	Room temp.	None

Table 10: Cationisation parameters.

Recombinant preparation of supercharged proteins

Preparing supercharged fusion proteins

Supercharged GFP was as described in Lawrence *et al*. (*J. Am. Chem. Soc*. (2007) vol. 129 p.10110-10112). For preparation of scGFP fusion proteins with the fibronectin-binding portion of CshA (SEQ ID NO:19), OpdA (SEQ ID NO:20), PlGF-$2_{(123-144)}$ (SEQ ID NO:21) and SpyCatcher (SEQ ID NO:23), a linker region was designed as outlined below. Subsequent steps were carried out as described above in the section headed "Plasmid preparation".

Linker design

The linking regions used to form the fusion proteins were designed using methods outlined in Chen *et al*. (*Adv. Drug. Deliv. Rev*. (2013) September 29;65,1357–69).

Supercharging OpdA

OpdA was supercharged to form scOpdA by mutation of 11 aspartic/glutamic acid residues to lysine residues, listed in Table 11 below (position numbering with reference to SEQ ID NO:10). The gene with mutated residues was ordered from Eurofins Genomics (Germany).

scOpdA modifications										
D76K	D97K	D109K	E120K	E121K	E135K	D136K	D184K	D211K	D212K	E239K

Table 11: Mutations made to OpdA to produce scOpdA

Protein-specific assays

Paraoxon hydrolysis

Proteins (OpdA-based constructs) were diluted to a working concentration in buffer. 100× paraoxon stocks were prepared in isopropanol. Formation of 4-nitrophenolate was measured at 405 nm, using an empirically determined extinction coefficient of ε_{405} = 12013 $M^{-1}{\cdot}cm^{-1}$. Non-linear regression was performed on initial-rate data to determine the Michaelis–Menten parameters.

[cOpdA][S] membrane activity assay

3′,6′-bis(diphenylphosphinyl) fluorescein (DPPF) was synthesised as a substrate for fluorescence imaging as described by Liguo An et al. (Chem. Eur. J. (2007), February 2;13:1411). DPPF was dissolved in DMSO to a stock concentration of 100 mM, and applied to cells at a final concentration of 1 mM for 30 minutes. Cells plated on glass bottom microwell dishes were labelled with 12 μM [cOpdA][S] after DPPF exposure. Images were collected using confocal microscopy. Acetylthiocholine was also used to assay the activity of membrane-bound [cOpdA][S] over 5 days. 450 μM acetylthiocholine and 300 μM 5,5'-dithiobis-(2-nitrobenzoic acid) was applied to hMSCs and hMSCs primed with 10 μM [cOpdA][S] plated in a 96-well plate at day 0, 1, and 5. The resulting absorbance was read at 412 nm over time, and an extinction coefficient of 14150 $M^{-1}.cm^{-1}$ was used to calculate acetylthiocholine turnover from the initial rate.

OpdA neutron reflectometry

Neutron reflectometry was performed on the INTER beamline at the Isis facility, Oxford, and on the D17 beamline at the Institut Laue–Langevin, Grenoble. Floating bilayers of 4:1 2-oleoyl-1-palmitoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (POPG) were assembled on a silicon block with a 1-Palmitoyl-2-[16-(acryloyloxy)hexadecanoyl]-sn-glycero-3-phosphorylcholine (al-PC) monolayer covalently bound to the silicon substrate using the Langmuir–Blodgett trough deposition method. OpdA constructs were loaded at concentrations of 0.2 and 5 μM, and loosely bound material washed with buffer. Data deconvolution was performed with the RasCal software package for MATLAB.

cThrombin fibrin formation

Thrombin-catalysed fibrin formation was measured through absorbance at 600 nm during the cationisation process. Briefly, 75 µL of 0.06 mg/mL thrombin was added to 125 µL of 5 mg/mL fibrinogen, shaken for 20 seconds, and the absorbance at 600 nm measured over time.

Fibrin constructs catalysed by membrane-bound [cThrombin][S]

[cThrombin][S]-primed hMSCs were added to a solution of 7.5 mg/mL human fibrinogen in wells precoated with agarose. Successful fibrin formation could be analysed using confocal microscopy with Alexa-594 tagged fibrinogen.

Solution coupling of SpyCatcher and SpyTag constructs

The coupling of [scGFP–SpyCatcher] or [scGFP–SpyCatcher][S] with either [mCherry–SpyTag] or [CshA–SpyTag] was investigated using SDS-PAGE. Equal volumes of either [scGFP_SpyCatcher] or [scGFP_SpyCatcher][S] and [mCherry_SpyTag] or [CshA_SpyTag] were mixed in a glass vial and agitated using a magnetic stirrer. At predetermined time points throughout the reaction, 10 uL of the resulting solution was removed and mixed with an equal volume of SDS sample application buffer for 5 minutes. The range of samples obtained in this method were applied to SDS-PAGE gels as previously described before subsequent staining and destaining.

Static adhesion assay for scGFP–CshA and scGFP–PlGF$_{(123-144)}$

Cell-substrate adhesion was investigated using a CyQUANT® NF cell proliferation assay kit (Invitrogen, UK). Human fibronectin (Sigma Aldrich) was diluted to 10 µg/mL with PBS, collagen I (rat tail; Sigma Aldrich) and collagen II (bovine trachea; Sigma Aldrich) were diluted to 0.2 mg/mL. 100 µL of these solutions were used to coat each well of the non-tissue-culture-treated 96 well plate. The plates were then washed three times with PBS solution containing 10 mg/mL bovine serum albumin (BSA; Sigma Aldrich) to block the non-specific interactions. The wells treated with BSA were used as a control. Cells were primed with protein complexes and the cells were harvested and counted using a haemocytometer. Standard curve samples were established in expansion medium. After four hours of incubation, medium was removed from cells by gentle aspiration and 100 µL of dye binding solution was dispensed into each well. The plate was covered and incubated at 37°C for 1 hour. The fluorescence intensity of each sample was measured using a plate reader with excitation at 485 nm and emission detection at 530 nm. Adhesive cell numbers were compared to control samples of untreated cells incubated with phenol-free DMEM.

Flow adhesion assay

Dynamic cell adhesion experiments were carried out with an ExiGo microfluidics pump (Cellix Ltd flowing through a Vena8 Fluoro+ biochip. The chip was coated overnight with 0.1 mg/mL collagen II (Sigma Aldrich) and unspecific sites were blocked with 10 µg/mL BSA

(Sigma Aldrich). The channel was washed with phenol-free DMEM with no additives for 30 seconds at 40 µL/min. scGFP–PlGF$_{(123-144)}$-primed and [scGFP–PlGF$_{(123-144)}$][S]-primed hMSCs were resuspended at a density of 1 million cells per mL in phenol-free DMEM without additives. A 50 µL aliquot was added to the channel reservoir each time and the cells were withdrawn at flow rates of 6, 4, or 3 mL/minute.

Adhesion to bovine articular cartilage explants

Cartilage explants were harvested form the lateral and patellar groove of 6–8-week old calves, obtained 6–8 hours after death. The disks were delimited with an 8mm biopsy punch and carefully detached with a surgical scalpel (size 22; Swann Morton). After dissection, the pieces were kept in DMEM with 10% FBS, 100 µg/mL penicillin-streptomycin. Cartilage discs were cut to 6 mm diameter with a biopsy punch and placed in a non-tissue culture treated 96 well plate (Fisher, UK) with 200 uL of phenol-free DMEM without supplements. Cells were primed with either scGFP–PlGF$_{(123-144)}$ or the corresponding conjugate and resuspended in phenol-free DMEM. Cells were added onto the cartilage and placed in incubator at 37°C with 5% CO2 for 4 hours. The samples were then fixed for SEM imaging or histology analysis.

In vivo transplantation of scGFP–CshA-primed hMSCs in mice

Male 20-week-old FVB/N and BALB/c nude mice were purchased from the Animal Resource Centre (Perth, Western Australia). All animal procedures were approved by the Animal Ethics Committee of the University of Queensland and were carried out in accordance with Australian Code for the Care and Use of Animals for Scientific Purposes 8th edition. Mice were anaesthetized with isoflurane. Body temperature was controlled by placing mice on a heating pad set to 37°C. 150 µL of a suspension of [scGFP–CshA][S], 2×10^6 untreated hMSCs, or [scGFP–CshA][S] modified hMSCs was injected with a 27 gauge needle either through a tail vein (intravenous injection) or through the chest wall into the left ventricle (intracardiac injection), respectively. Prior to the injection, the hMSCs were maintained at 4°C, and the cells were gently resuspended with a pipette to ensure no aggregation before the injection. The mice were sacrificed at 2 hours and 24 hours after the injection. Genomic DNA of the heart and lung were isolated using DNA Mini Kit (Qiagen, USA) and primers targeting the human *Alu* sequence according to the manufacturer's instructions. Droplet digital PCR (ddPCR) was then used to quantify the number of human cells in each tissue. Briefly, 20µL of ddPCR reaction mix was separated into droplets with a QX200 Droplet Generator (BioRad, USA). The droplets were transferred into a 96-well PCR plate, sealed and incubated at following cycling conditions: one cycle of 95^{o}C for 5 minutes, 45 cycles of 95^{o}C for 30 seconds, 55^{o}C for 1 minute and one cycle of 4^{o}C for 5 minutes, 90^{o}C for 5 minutes and an infinite hold of 12^{o}C. After thermal cycling, the PCR plate was transferred in QX200 Droplet Reader (read) and read in the FAM channel using QuantaSoft version 1.7.

Results

Protein expression and purification

All proteins were confirmed to be expressed and purified using SDS–PAGE, mass spectrometry (MADLI–TOF), and activity assays. The mass spectrum for scGFP–OpdA is shown in Figure 5. UV–visible spectrophotometry and fluorescence spectrophotometry confirmed the correct folding of the scGFP-fusion constructs (Figures 6–8).

Cationisation

The successful cationisation of OpdA and thrombin was confirmed using matrix-assisted laser-desorption-ionisation time-of-flight mass spectrometry (MALDI–TOF). OpdA was shown to increase in mass by approximately 1700 Da, corresponding to the addition of 20 DMPA molecules. Thrombin cationisation led to an increase in mass of approximately 3300 Da, equivalent to 39 DMPA molecules. The mass spectra collected at a charge number of 3 for thrombin are shown in Figure 9.

Zeta potentiometry was used to show the increased charge associated with cationisation. Cationisation increased the zeta potential of OpdA from -7 mV to +21 mV (see Figure 10). The change in zeta potential during cationisation of thrombin is shown in Figure 11.

Structural changes associated with cationisation were assayed using dynamic light scattering (DLS), circular dichroism (CD), and small angle X-ray scattering (SAXS). DLS showed the cationisation of OpdA lead to an increase in size of 0.8 nm corresponding to the addition of DMPA molecules to surface residues, whilst CD showed minimal changes in secondary structure but an increase in thermal stability (Figure 12). SAXS showed OpdA remained dimeric post-cationisation (Figure 13).

Conjugation

Electrostatic grafting of the anionic headgroup of the surfactant to positively charged residues leads to a decrease in the surface charge of proteins, therefore the zeta potential is expected to decrease. scGFP–PlGF$_{(123-144)}$ was shown to have a zeta potential of +22 mV, while [scGFP–PlGF$_{(123-144)}$][S] was -0.5 mV. scGFP–CshA had a zeta potential of +1 mV despite the highly anionic CshA region, and [scGFP–CshA][S] was -15 mV. cOpdA to [cOpdA][S] showed a reduction of 13 mV (Figure 10).

An increase in size corresponding to the addition of a surfactant corona is also expected. DLS showed an increase in hydrodynamic diameter of 1.9 nm, 2nm and 2.9 nm for the conjugation of cOpdA, scGFP–CshA and scGFP–PlGF$_{(123-144)}$, respectively. scGFP–OpdA showed a 388 nm increase in size due to the formation of clusters. SV–AUC showed an increase in the sedimentation coefficient of [scGFP–CshA][S] from 4.1 to 4.8, indicating surfactant binding.

Importantly, surfactant conjugation did not lead to denaturation. CD was used to assess the secondary structures of OpdA and scGFP–OpdA constructs. [cOpdA][S] showed minimal changes in secondary structure to each of OpdA and cOpdA, and retained the improved thermal stability of cOpdA (Figure 12). Conjugation of scGFP–OpdA lead to an increase in thermal stability (Figure 14). UV–visible spectrophotometry and fluorescence spectrophotometry confirmed that the fluorophore's structure was maintained (Figure 6).

Cell loading

The successful loading of the conjugate systems to membranes was confirmed through microscopy, spectrophotometry, flow cytometry, reflectometry and activity assays.

The scGFP-based constructs are inherently fluorescent, and so were simply visualised using fluorescence microscopy. scGFP–CshA, [scGFP–CshA][S], scGFP–$PlGF_{(123-144)}$, [scGFP–$PlGF_{(123-144)}$][S], scGFP–OpdA, and [scGFP–OpdA][S]-primed cells all displayed fluorescent membranes, but the conjugate species retained the membrane fluorescence for a longer time period: approximately 15 minutes for unconjugated protein versus more than 24 hours for conjugated protein, indicating that the unconjugated proteins are rapidly endocytosed, whilst conjugated protein is retained at the membrane.

Thrombin and OpdA each had to be fluorescently tagged prior to imaging, as described in 'Microscopy' method section. cOpdA was rapidly internalised, whereas [cOpdA][S] remained at the cell membrane. [cThrombin][S] was observed at the cell membrane for up to 7 days. Native OpdA and thrombin did not interact with cells.

UV–visible spectrophotometry may be used to determine the amount of protein bound to cell membranes. Approximately 0.7 billion scGFP–CshA complexes bound per cell, whereas 1 billion [scGFP–CshA][S] complexes bound per cell, after 15 minutes (Figure 15). Similarly, flow cytometry showed significantly greater fluorescence for [scGFP–CshA][S]-loaded cells versus scGFP–CshA -loaded cells (Figure 16). The amount of scGFP–CshA observable per cell is significantly reduced after longer time periods compared to the amount of [scGFP–CshA][S], due to rapid endocytosis of the unconjugated protein.

Neutron reflectometry was used to assess the insertion of [cOpdA][S] into a model membrane. [cOpdA][S] was shown to insert into the lipid bilayer and, once the membrane was saturated with conjugate, forms a layer above the membrane. cOpdA penetrated the bilayer and disrupted the supporting monolayer, indicating that it was not embedded within the membrane.

Importantly, the cells could be treated with each protein construct without significant cell cytotoxicity. The surfactant, glycolic acid ethoxylate 4-nonylphenyl ether, was also assayed. The maximum assayed loading concentrations below significant cytotoxicity are

presented in Table 12 below. Asterisks mark the data where significant cytotoxicity was not observed. The data for scGFP–CshA and [scGFP–CshA][S] are shown in Figure 17.

Primed cells were also shown to proliferate readily. [cThrombin][S]-primed cells were shown to proliferate via an MTS assay over 22 days (Figure 11). scGFP–CshA and [scGFP–CshA][S]-primed cells proliferated at the same rate as unprimed cells, as confirmed by direct cell counting (Figure 18) , indicating that process of priming the cells with the priming entity (scGFP-CshA or [scGFP-CshA][S]) had no impact on the ability of cells to proliferate.

The differentiation potential of cells was also not affected by priming. [cThrombin][S]-primed cells treated with adipogenic or osteogenic media displayed characteristic fat droplets and extracellular calcium deposits, respectively, after 21 days, as observed with widefield microscopy.

Protein	**Maximum assayed loading concentration below significant cytotoxicity**	**Assay method**
[cOpdA][S]	15 μM	alamarBlue
[cThrombin][S]	5.2 μM	MTS
[scGFP–CshA][S]	8 μM	MTS
[scGFP–PlGF$_{(123-144)}$][S]	12 μM	MTS
[scGFP–SpyCatcher][S]	14 μM	MTS
mCherry–SpyTag	14 μM	MTS
[scGFP–SpyCatcher][S]–[mCherry–SpyTag]	14 μM	MTS
Glycolic acid ethoxylate 4-nonylphenyl ether	25 mM*	MTS

Table 12: Maximum loading concentrations for the conjugated proteins; * indicates significant cytotoxicity was not observed

Solution activity of constructs

Post-modification, each protein maintained activity. Previous work by Brogan *et al.* (Nat. Commun. (2014) October 10;5:5058) with lipases from *Rhizomucor miehei* and *Thermomyces lanuginosus* reported a 98% and 85% reduction in substrate-turnover rate post-cationisation, and a further 55% and 40% reduction post-conjugation, respectively, therefore it is surprising that the inventors were able to maintain activity. The assays required to determine activity are specific to each protein.

OpdA-based constructs were assayed for activity by measuring the hydrolysis rates of paraoxon. The Michaelis–Menten parameters of cOpdA were not significantly different to those of OpdA, however the K_M was significantly decreased (25.0 ± 4.49 vs. 45.6 ± 8.80

μM) and the k_{cat} significantly increased (92.4 ± 3.63 vs. 75.4 ± 3.68 s-1) for [cOpdA][S], leading to a 2.2-fold increase in the specificity constant (Figure 19). scGFP–OpdA was shown to have a K_M of 31 μM, and a k_{cat} of 1.98 s^{-1}.

The activity of thrombin was assayed by measuring the absorbance at 600 nm corresponding to fibrin formation from fibrinogen cleavage. During cationisation, the activity of thrombin was retained up to 120 minutes of cationisation, although the activity was gradually decreased with increased cationisation duration, as shown in Figure 20.

The coupling of scGFP–SpyCatcher and [scGFP–SpyCatcher][S] to either mCherry–SpyTag or CshA–SpyTag was assayed using SDS–PAGE. The appearance of a band at a high molecular weight indicated the formation of the isopeptide bond between the SpyTag and SpyCatcher moieties, in each of the conjugated and non-conjugated samples, for each of the SpyTag constructs.

Membrane activity of constructs

The activity of [cOpdA][S] at cell membranes was followed via microscopy. Cells exposed to DPPF for 30 minutes then treated with [cOpdA][S] exhibited increased fluorescence at cell membranes. Furthermore, hMSCs primed with [cOpdA][S] were able to turn over more acetylthiocholine than unprimed cells over at least 5 days.

[cThrombin][S] bound to cell membranes was able to cleave fibrinogen to form a fibrin gel, as confirmed with confocal microscopy, using Alexa-594-tagged fibrinogen (Figure 21).

scGFP–CshA, scGFP–$PlGF_{(123-144)}$, and their respective conjugates were assayed for their ability to bind to fibronectin (scGFP–CshA and scGFP–$PlGF_{(123-144)}$) and collagen I and II (scGFP–$PlGF_{(123-144)}$). scGFP–CshA-primed cells and [scGFP–CshA][S]-primed cells adhered in significantly greater numbers than unlabelled cells to fibronectin-treated plates (Figure 22). Under 4 mL/min flow, hMSCs primed with [scGFP–$PlGF_{(123-144)}$][S] adhered in significantly greater numbers to collagen II than unprimed hMSCs, as observed *via* widefield microscopy.

Flow cytometry showed that hMSCs primed with scGFP–SpyCatcher or its corresponding conjugate were able to form covalent bonds with mCherry–SpyTag for up to 72 hours.

[scGFP–$PlGF_{(123-144)}$][S]-primed hMSCs were seen to adhere in greater numbers to explanted bovine articular cartilage than unprimed hMSCs, as seen in Figure 23.

In vivo activity of constructs

[scGFP–CshA][S]-primed hMSCs were transplanted into mice *via* intravenous and intracardiac injection. Upon harvesting the heart and lung tissue from the mice after 2 hours, 24 hours, and 4 weeks, the number of hMSCs in each tissue was determined using

droplet digital PCR. The tissue:plasma distribution coefficient of [scGFP–CshA][S]-primed hMSCs in the heart was shown to have increased 2-fold relative to unprimed hMSCs at 2 hours and 24 hours.

CLAIMS

1. A phospholipid composition which is a bilayer or micelle comprising at least one embedded protein-polymer surfactant conjugate comprising an anchor protein, wherein the anchor protein is a cationised protein or an anionised protein, the composition characterised in that the anchor protein is:

    a. an enzyme; or

    b. is a protein which does not comprise a $-CH_2C(O)NCH_3(CH_2)_3N(CH_3)_2H^+$ linker covalently bonded to an amino acid side chain.

2. A composition according to claim 1, wherein the protein-polymer surfactant conjugate comprises a surfactant containing polyethylene glycol.

3. The composition of claim 1 or 2, wherein the protein-polymer surfactant conjugate comprises a surfactant having a molecular weight of at least about 500 Da, optionally wherein the surfactant is S1783.

4. The composition of any preceding claim, wherein the phospholipid bilayer forms the surface membrane of a cell, optionally a mesenchymal stem cell or a cardiomyocyte.

5. The composition of any preceding claim, wherein the anchor protein is a protein which does not comprise a $-CH_2C(O)NCH_3(CH_2)_3N(CH_3)_2H^+$ linker covalently bonded to an amino acid side chain and wherein the anchor protein is linked to a secondary molecule which is CshA or is a functional variant or portion of CshA comprising SEQ ID NO:19.

6. A pharmaceutical composition comprising the phospholipid composition according to any of claims 1-5, further comprising a pharmaceutically acceptable carrier, diluent or vehicle.

7. A surgical composition comprising a phospholipid composition according to any of claims 1-5 and at least one surgically acceptable carrier, diluent or vehicle.

8. A method of making a composition according to any preceding claim, comprising

    a. providing a protein-polymer surfactant conjugate; and

    b. contacting a phospholipid bilayer or micelle with the conjugate;

wherein the protein-polymer surfactant conjugate comprises an anchor protein which is a cationised protein or an anionised protein and (i) is an enzyme and/or (ii) is a protein

which does not comprise a $-CH_2C(O)NCH_3(CH_2)_3N(CH_3)_2H^+$ linker covalently bonded to an amino acid side chain.

9. The method of claim 8, wherein the phospholipid bilayer is a cell and step (b) comprises contacting the cell with the conjugate and incubating at a temperature of at least about 10°C for a period of at least about 2 minutes, optionally followed by a step of washing the cell.

10. The method of claim 8 or 9, wherein step (a) comprises contacting an anchor protein which is a cationised protein or an anionised protein with a surfactant under conditions which enable electrostatic conjugation of the surfactant with the protein.

11. The method of claim 10, wherein the surfactant contains polyethylene glycol.

12. The method of claim 10 or 11 wherein the surfactant has a molecular weight of at least about 500 Da, optionally wherein the surfactant comprises S1783.

13. The method of claim 10, 11 or 12 wherein the anchor protein is obtained by a method comprising:

i. mixing a solution of an anchor precursor protein with a pH-neutralised solution of N,N'-dimethyl-1,3-propanediamine (DMPA) or analogue thereof and optionally adjusting the mixture to pH 5-7;

ii. subsequently or concurrently adding a carbodiimide such as N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and adjusting the mixture to pH 4-7;

iii. agitating the mixture from (ii) for 1-30 hours at pH 4-7, at a temperature of 0-25°C;

iv. dialysing the protein in the mixture from (iii) against water or buffer for at least 4 hours at pH 6.5-8.5;

v. if necessary, adjusting the pH of the mixture from (iv) to pH 6.5-8.5;

wherein the anchor precursor protein is an enzyme; further wherein:

- step (iii) continues for no longer than about 120 minutes; and/or
- the method further comprises a step (vi) of size exclusion chromatography of the mixture from step (iv), or from step (v) when present.

14. The method of claim 10, 11 or 12 wherein the anchor protein is obtained by a method comprising expression of a recombinant DNA sequence encoding for a supercharged protein.

15. The method of claim 14 wherein the recombinant DNA sequence encodes for a fusion protein comprising the supercharged protein and a secondary molecule.

16. The method of claim 15 wherein the secondary molecule comprises one or more of CshA, a functional variant or portion of CshA comprising SEQ ID NO:19, OpdA (SEQ ID NO:10 or SEQ ID NO:39), thrombin, prothrombin, PlGF-2 (SEQ ID NO:22), a functional variant or portion of PlGF-2 comprising SEQ ID NO:21, a SpyCatcher polypeptide (SEQ ID NO:23) or a SpyTag polypeptide (SEQ ID NO:24), or comprises a functional variant of any of these having at least about 60% sequence identity to the non-variant sequence.

17. The method of any of claims 14-16 wherein the recombinant DNA sequence has sequence SEQ ID NO:4-7.

18. A method of labelling a cell with a protein label, comprising a method according to any of claims 8-17, wherein the phospholipid bilayer forms the external membrane of the cell and the protein-polymer surfactant conjugate comprises the protein label.

19. The method according to claim 18 wherein step (a) of the method of claim 8 comprises contacting an anchor protein which is a cationised protein or an anionised protein with a surfactant under conditions which enable electrostatic conjugation of the surfactant with the protein, further wherein the anchor protein is obtained by a method comprising expression of a recombinant DNA sequence encoding for a fusion protein comprising a supercharged protein and a secondary molecule, wherein the protein label is the secondary molecule.

20. A tissue engineering scaffold comprising a phospholipid composition according any of claims 1-5.

21. A tissue engineering scaffold according to claim 20, wherein the phospholipid composition comprises an anchor protein which is, in its anchor precursor protein form, known to promote growth and/or healing of tissue, and/or wherein the phospholipid composition comprises a secondary molecule which is known to promote growth and/or healing of tissue.

22. The tissue engineering scaffold according to claim 21 wherein the anchor protein is cationised or supercharged thrombin, or is a cationised or supercharged functional variant of thrombin, or is a cationised or supercharged functional variant of

prothrombin, and/or wherein the secondary molecule is thrombin, prothrombin, PlGF-2 (SEQ ID NO:22), or a functional variant or portion of PlGF-2 comprising SEQ ID NO:21.

23. A method for forming a tissue engineering scaffold according to claim 21 or 22 comprising contacting a cell according to claim 4 in which the anchor protein is cationised or supercharged thrombin or prothrombin or is a cationised or supercharged functional variant of thrombin or prothrombin, or in which the protein-polymer surfactant conjugate comprises a secondary molecule which is thrombin or prothrombin or a functional variant of thrombin or prothrombin, with a fibrinogen composition such as a fibrinogen-containing gel or structure.

24. A method of promoting tissue growth and/or healing, comprising use of a cell which is a phospholipid composition according to claim 4 in which the anchor protein is known to promote growth and/or healing of the tissue, or in which the protein-polymer surfactant conjugate comprises a secondary molecule which is known to promote growth and/or healing of the tissue, by introducing the cell to a site where the tissue is desired to grow and/or heal.

25. The method according to claim 24 wherein the cell is a mesenchymal stem cell and the protein-polymer surfactant conjugate comprises a secondary molecule which is PlGF-2 (SEQ ID NO:22) or a variant or portion thereof comprising SEQ ID NO:21.

26. A method according to claim 24 or 25 comprising use of the pharmaceutical composition according to claim 6 or the surgical composition according to claim 7 or the tissue engineering scaffold according to claim 20.

27. A method of targeting a cell to a tissue, comprising use of a cell which is a phospholipid composition according to claim 4, wherein the phospholipid bilayer forms the external membrane of the cell and the protein-polymer surfactant conjugate comprises a protein specific for the tissue.

28. The method according to claim 27 wherein the protein specific for the tissue is the anchor protein or is the secondary molecule.

29. The method according to claim 28 wherein the secondary molecule is a cationised or supercharged antibody, lectin, integrin or adhesion molecule, optionally CshA (SEQ ID NO:20) or a portion or functional variant thereof comprising SEQ ID NO:19.

30. A method according to any of claims 27-29 comprising use of the pharmaceutical composition according to claim 6 or the surgical composition according to claim 7 or a tissue engineering scaffold according to claim 20.

31. A method according to any of claims 27-30 which is a method of treating a human or animal individual suffering from myocardial infarction, cardiomyopathy and/or myocarditis.

32. A method of delivering a protein to the interior of a cell, comprising use of the method according any of claims 8-17 to obtain a cell which is a phospholipid composition according to claim 4, wherein the phospholipid bilayer forms the external membrane of the cell, further wherein the protein-polymer surfactant conjugate comprises a molecule which promotes or inhibits the speed/rate of endocytosis.

33. The phospholipid composition according to any of claims 1-5 or the pharmaceutical composition according to claim 6 or the surgical composition according to claim 7 or a tissue engineering scaffold according to claim 20, for use in therapy.

34. The phospholipid composition according to claim 33, for use in promoting tissue growth and/or healing, wherein the composition comprises a protein known to promote growth and/or healing of the tissue.

35. The phospholipid composition according to claim 33 or 34 for use in a method of treatment of myocardial infarction, cardiomyopathy and/or myocarditis.

36. The phospholipid composition for use according to claim 33, for use in the treatment of a poisoned human or animal which has been contacted with a poison, wherein the phospholipid composition (a) comprises an anchor protein which is an enzyme which can neutralise the poison; or (b) comprises an anchor protein which is linked to a secondary molecule which can bind to or neutralise the poison.

37. The phospholipid composition for use according to claim 36, wherein the anchor protein which is an enzyme is cationised or supercharged OpdA or a cationised or supercharged functional variant or portion thereof capable of degrading an organophosphorus compound, or wherein the secondary molecule linked to the anchor protein is OpdA (SEQ ID NO:10 or SEQ ID NO:39) or a functional variant or portion thereof capable of degrading an organophosphorus compound.

38. A method of decontaminating a sample comprising a poison, comprising either: (a) contacting the sample with a composition according to any of claims 1-5, wherein the anchor protein is an enzyme which can neutralise the poison; or (b) contacting the sample with a composition according to any of claims 1-5, wherein the anchor protein is linked to a secondary molecule which can bind to or neutralise the poison.

39. The method of claim 38 wherein the sample is a liquid sample or a solid sample.

40. The method of claim 39 wherein the solid sample is a surface, land, a soil sample, or a fabric, optionally wherein the fabric is clothing or bedding.

41. The method of any of claims 38-40 wherein the anchor protein which is an enzyme is cationised or supercharged OpdA or a cationised or supercharged functional variant or portion thereof capable of degrading an organophosphorus compound, or wherein the secondary molecule linked to the anchor protein is OpdA (SEQ ID NO:10 or SEQ ID NO:39) or a functional variant or portion thereof capable of degrading an organophosphorus compound.

42. A polypeptide comprising any of SEQ ID NOs:11, 13-16 or 40 or a functional variant of any of these having at least about 60% sequence identity with the non-variant sequence; or

comprising a supercharged version of any of SEQ ID NOs:10, 19-22 or 25-39; or

comprising a fusion protein of: a supercharged anchor protein and any of SEQ ID NOs:10 or 19-39; or a supercharged anchor protein and a functional variant of any of any of SEQ ID NOs:10 or 19-39 having at least 60% sequence identity thereto.

43. A polynucleotide encoding a polypeptide according to claim 42, optionally comprising any of SEQ ID NOs:2 or 4-7.

44. A pharmaceutical composition comprising a polypeptide according to claim 42 and/or a polynucleotide according to claim 43.

45. A polypeptide according to claim 42 and/or a polynucleotide according to claim 43, or a pharmaceutical composition according to claim 44, for use in therapy.

46. A therapeutic method comprising administering a polypeptide according to claim 42 or 45, and/or the nucleic acid according to claims 43 or 45, or a pharmaceutical composition according to claim 44 or 45, to a subject requiring therapeutic treatment.

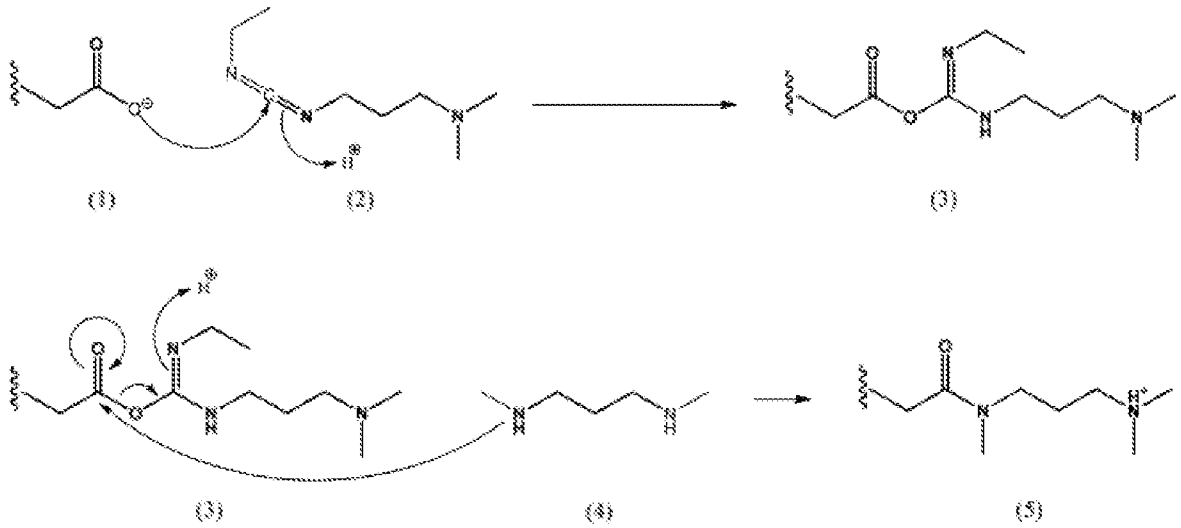


Figure 1

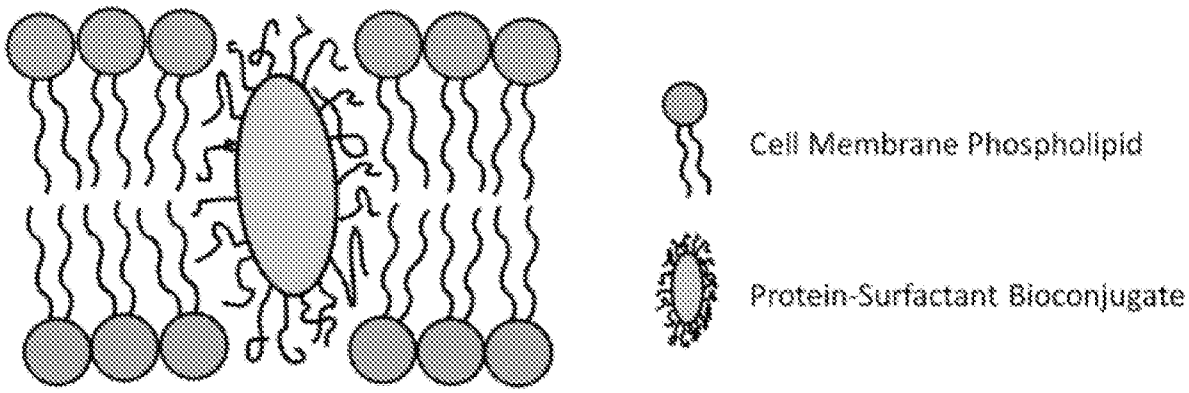


Figure 2

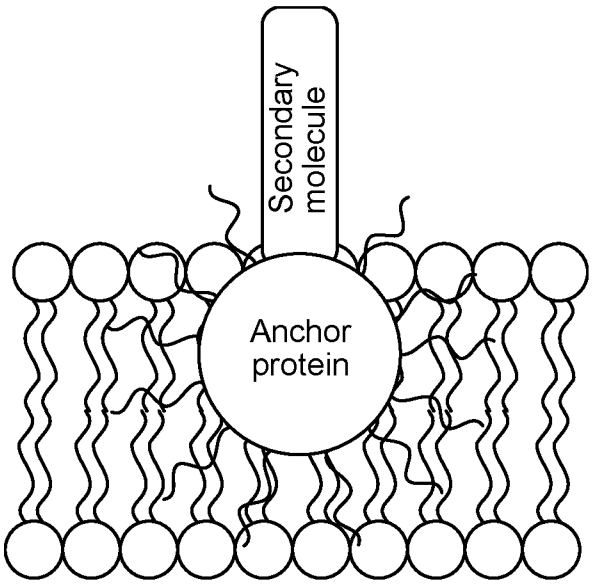


Figure 3

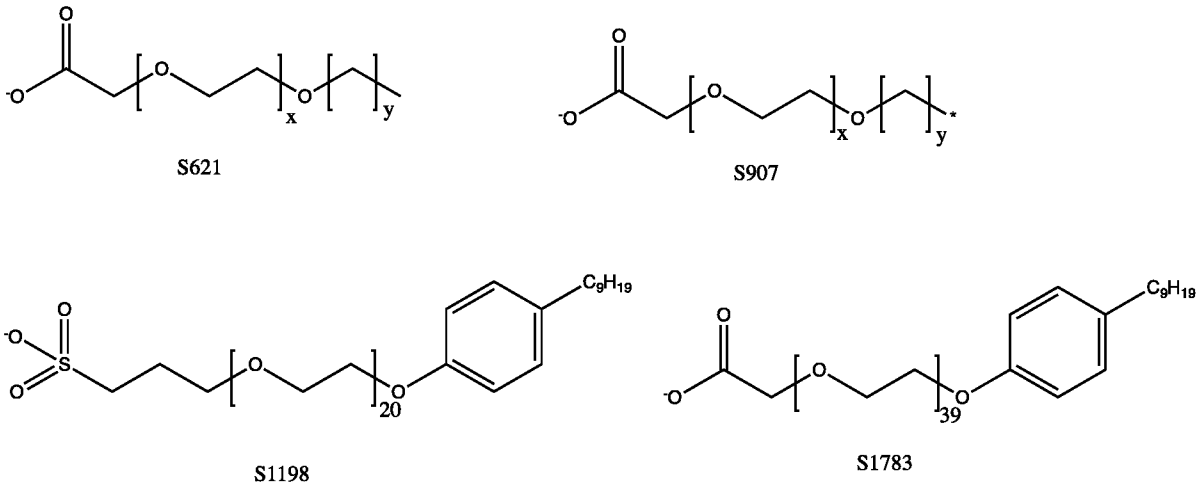


Figure 4

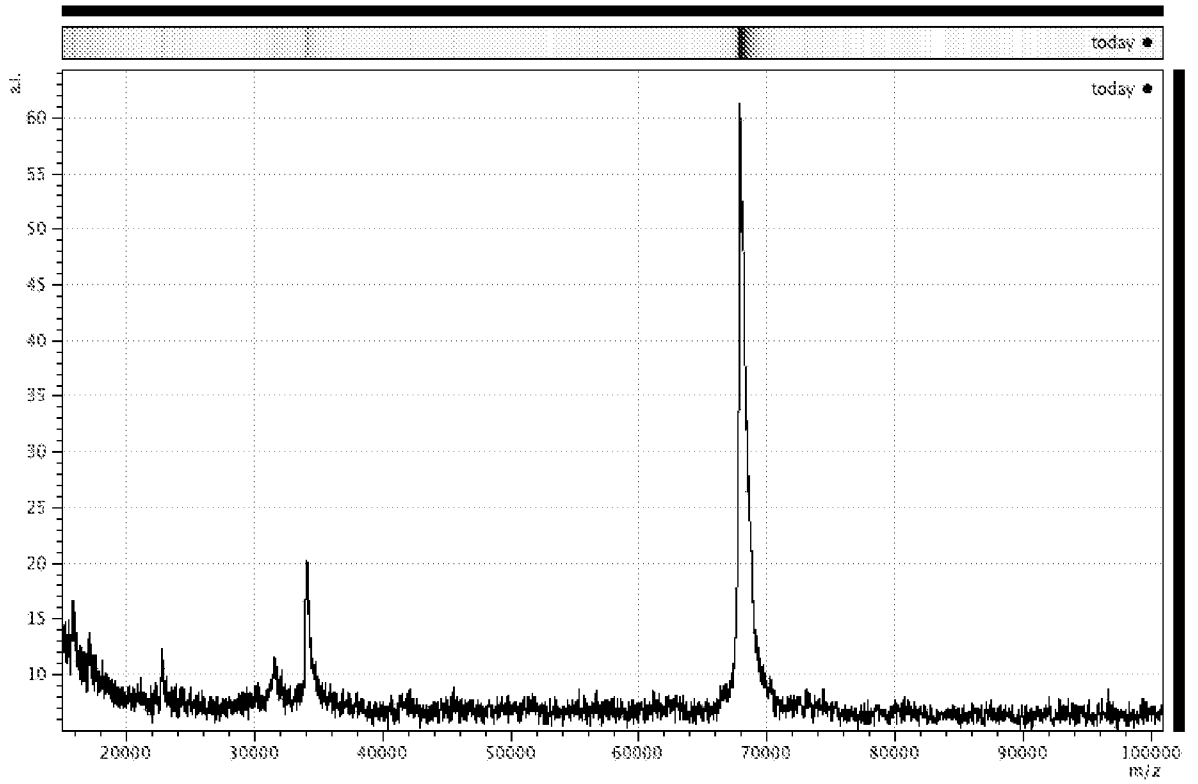
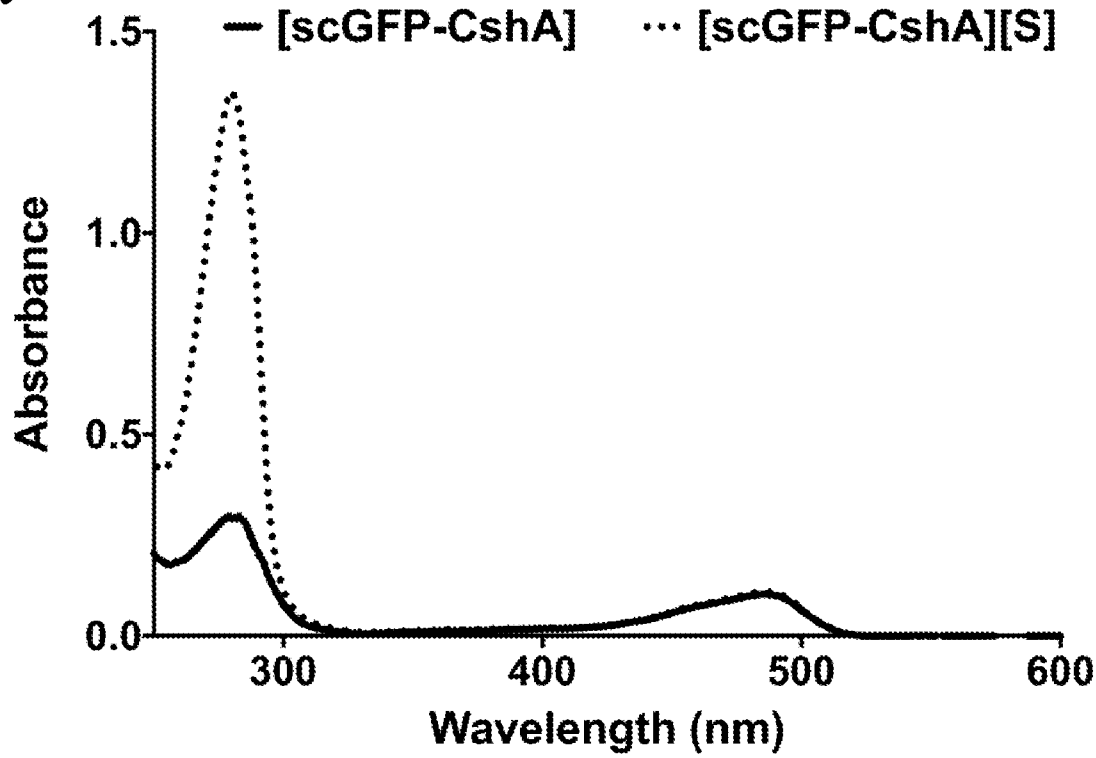


Figure 5

a


[scGFP-CshA]
[scGFP-CshA][S]
Absorbance
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1.0
0.5
0.0
300
400
500
600
Wavelength (nm)

b

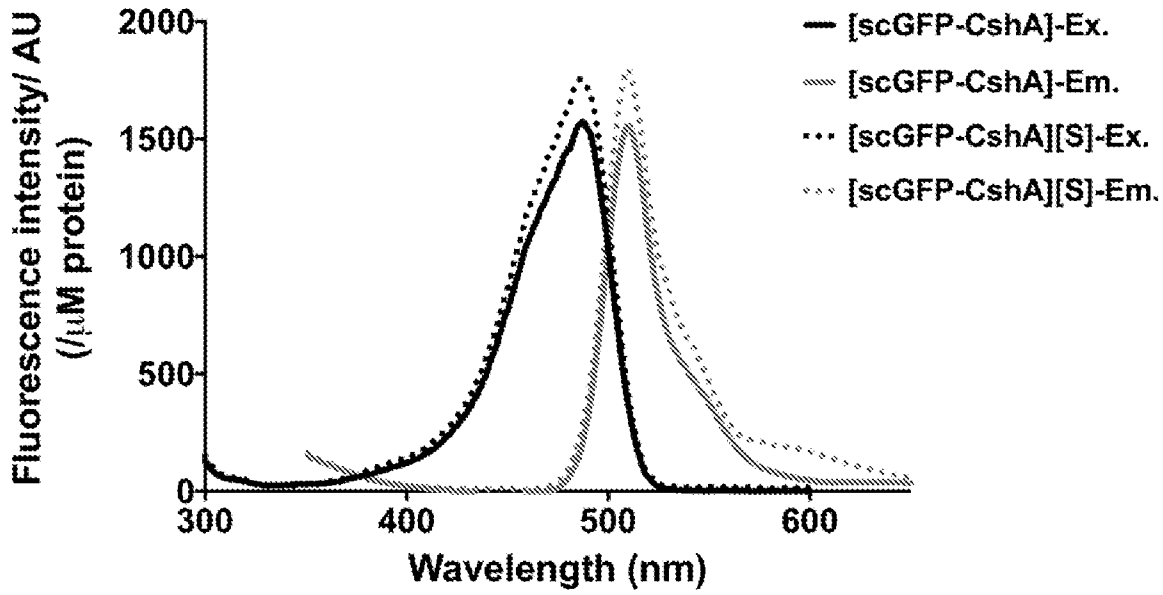
[scGFP-CshA]-Ex.
[scGFP-CshA]-Em.
[scGFP-CshA][S]-Ex.
[scGFP-CshA][S]-Em.
Fluorescence intensity/ AU (/μM protein)
2000
1500
1000
500
0
300
400
500
600
Wavelength (nm)

Figure 6

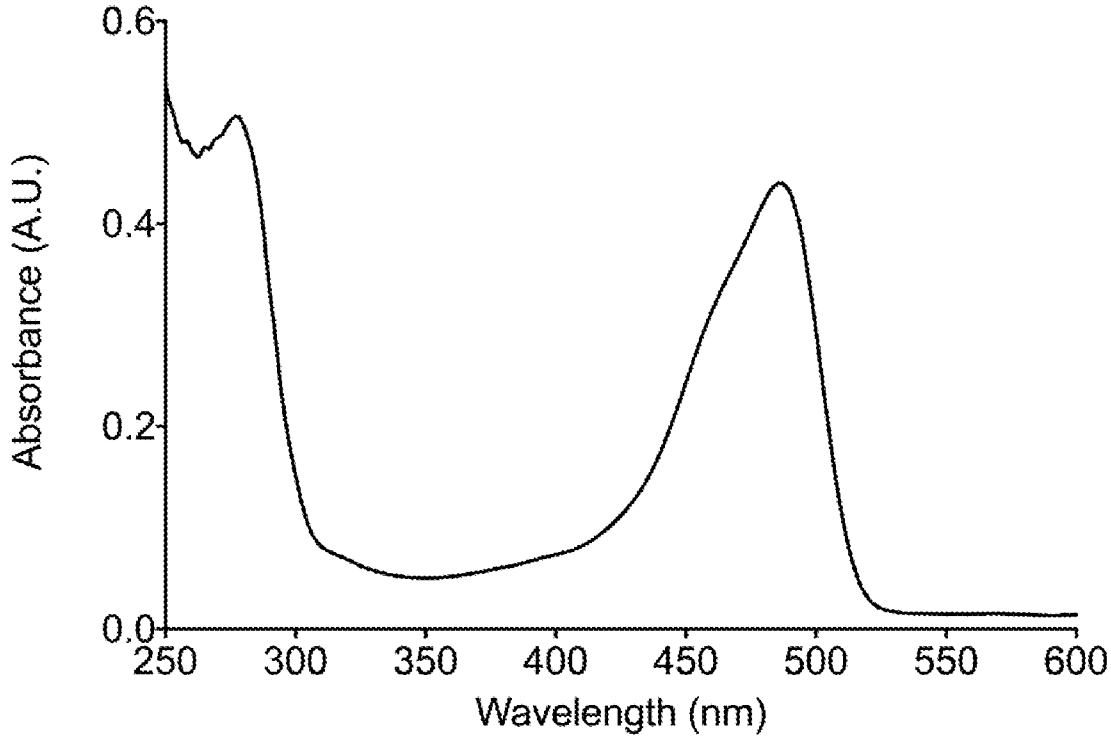

Figure 7

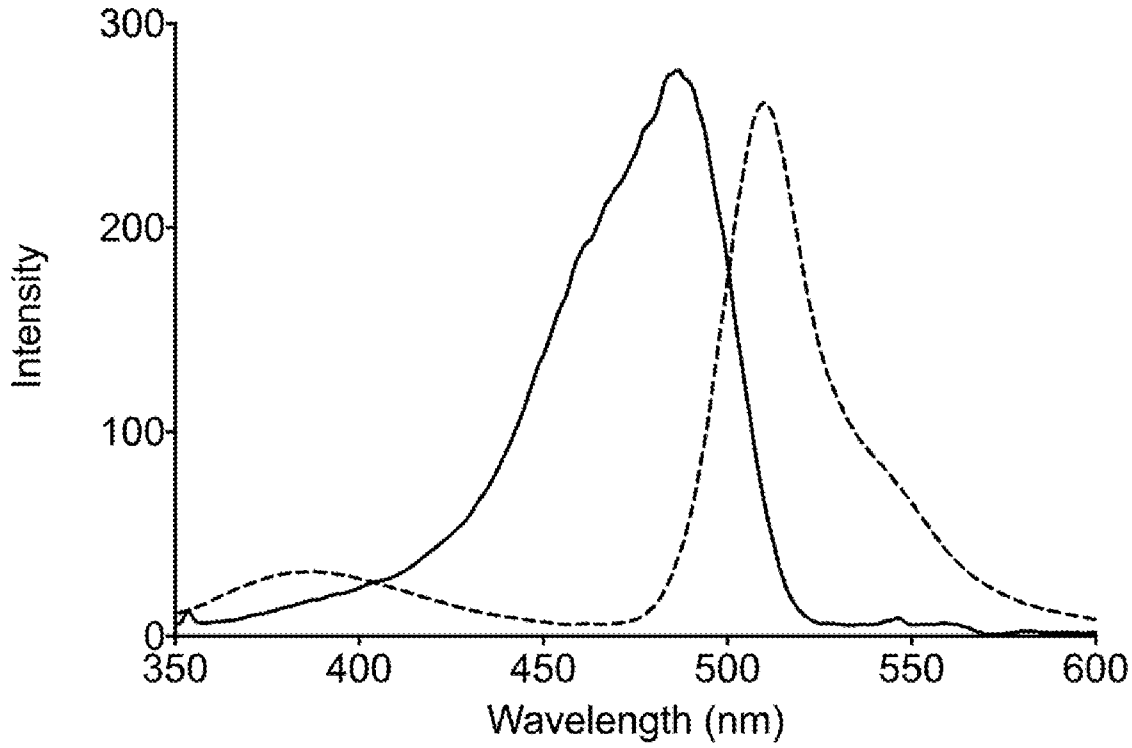

Figure 8

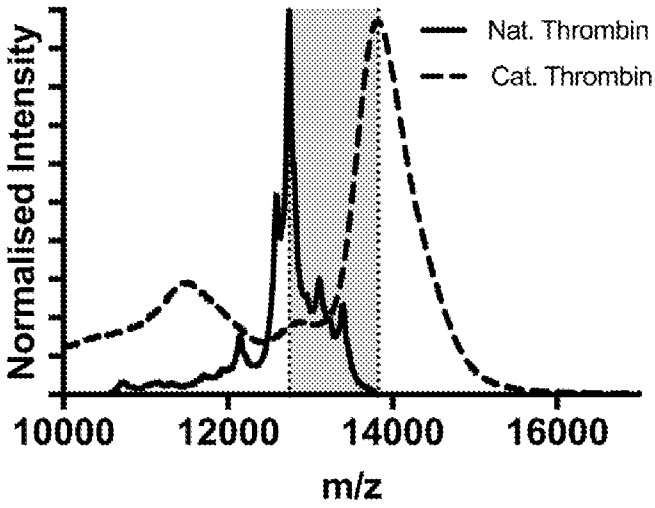

Figure 9

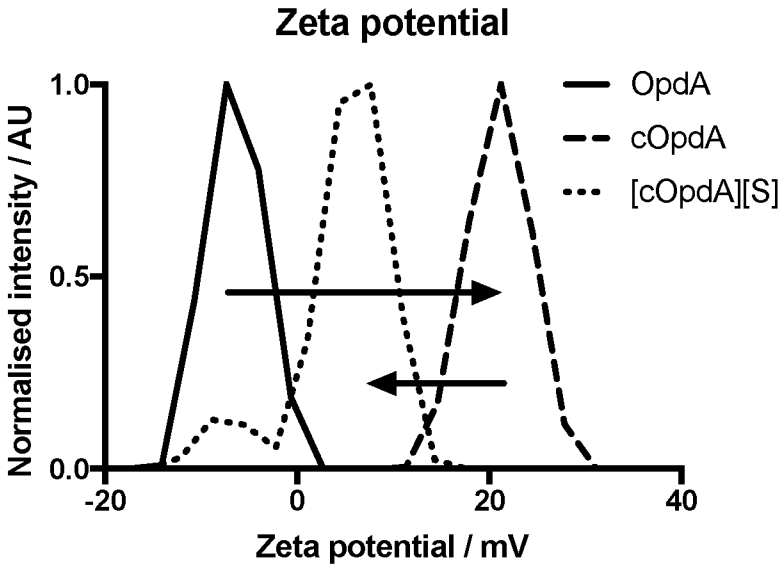

Figure 10

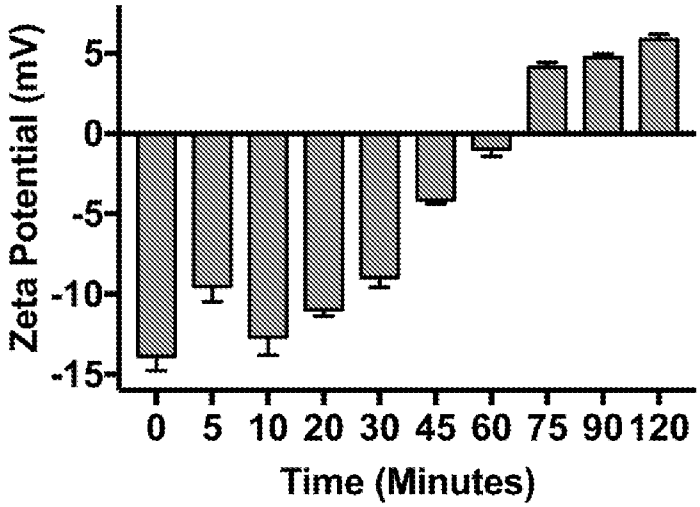

Figure 11

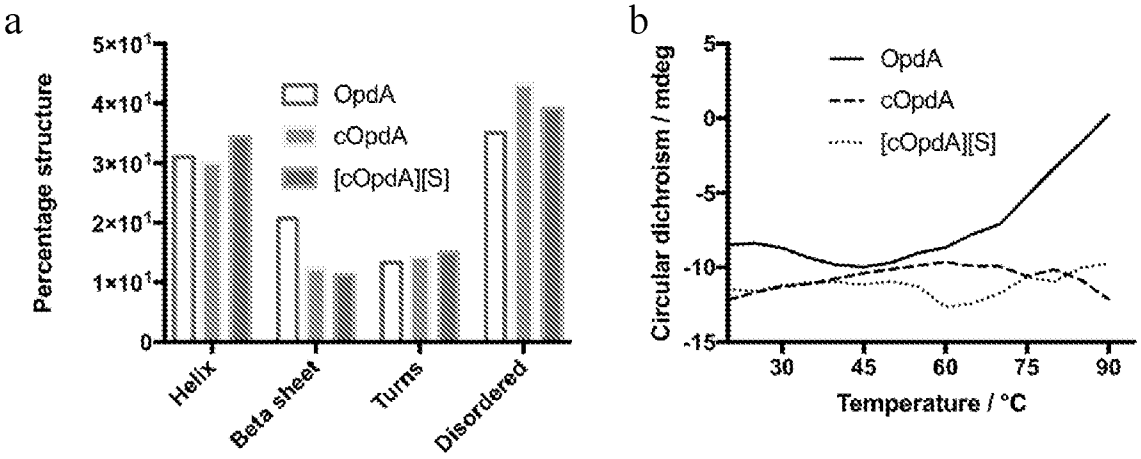

Figure 12

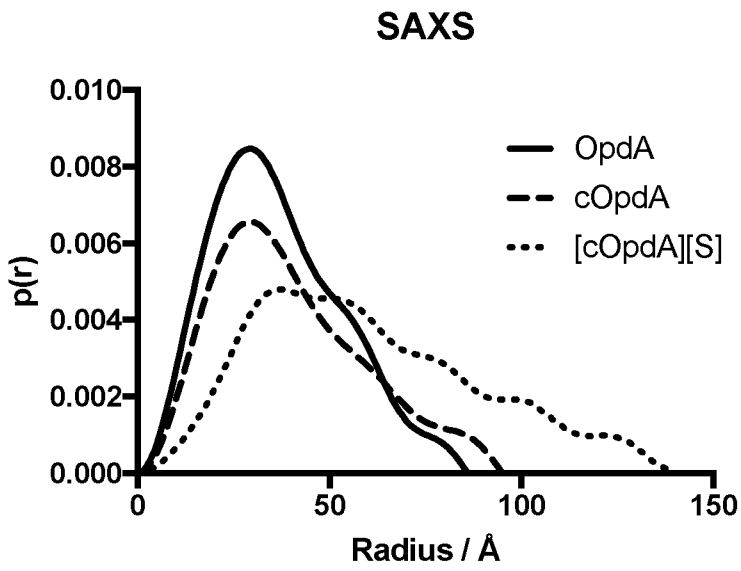

Figure 13

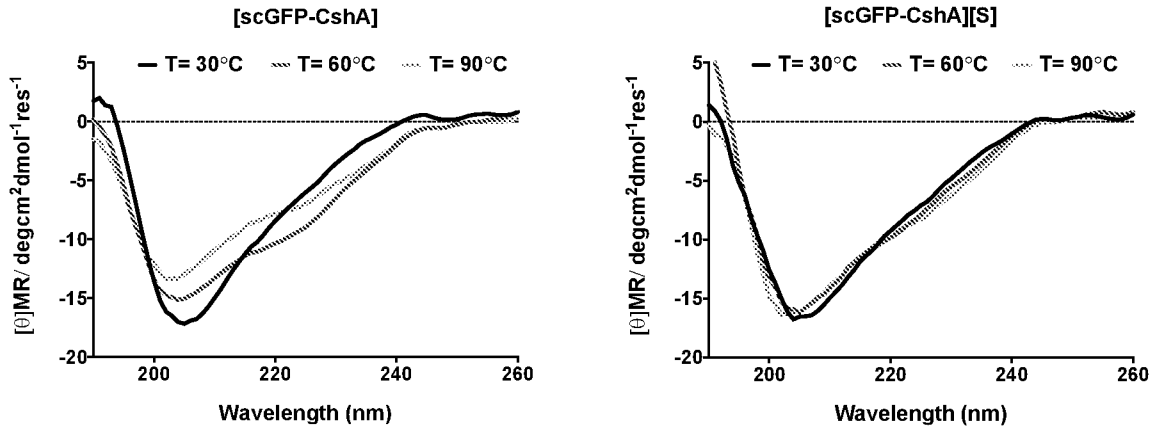

Figure 14

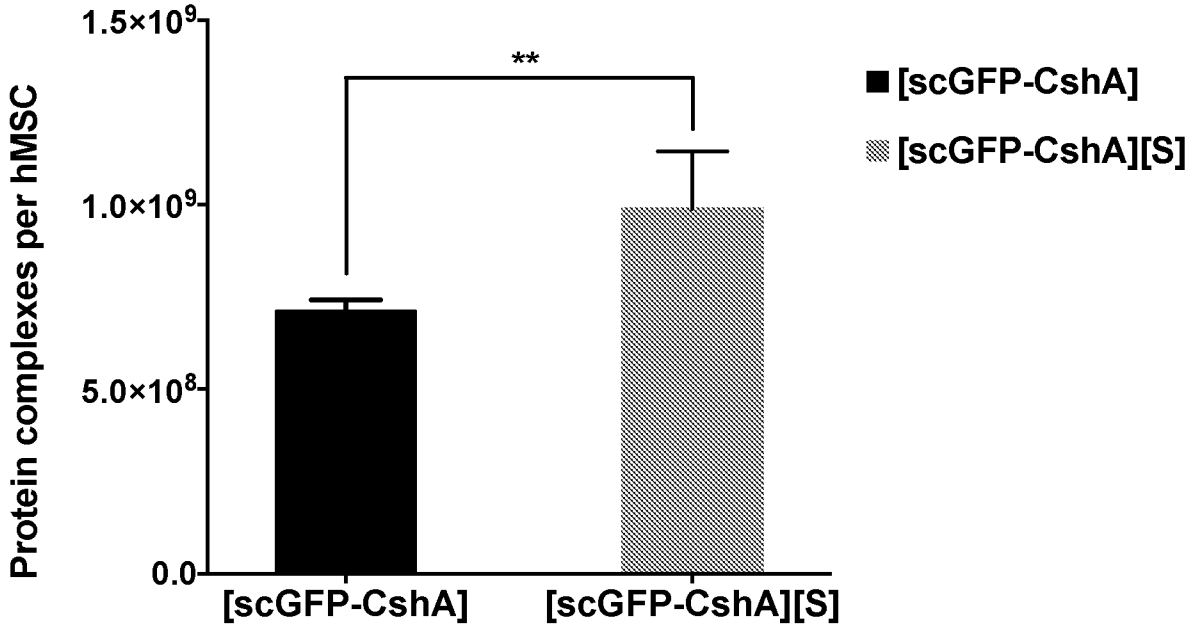

Figure 15

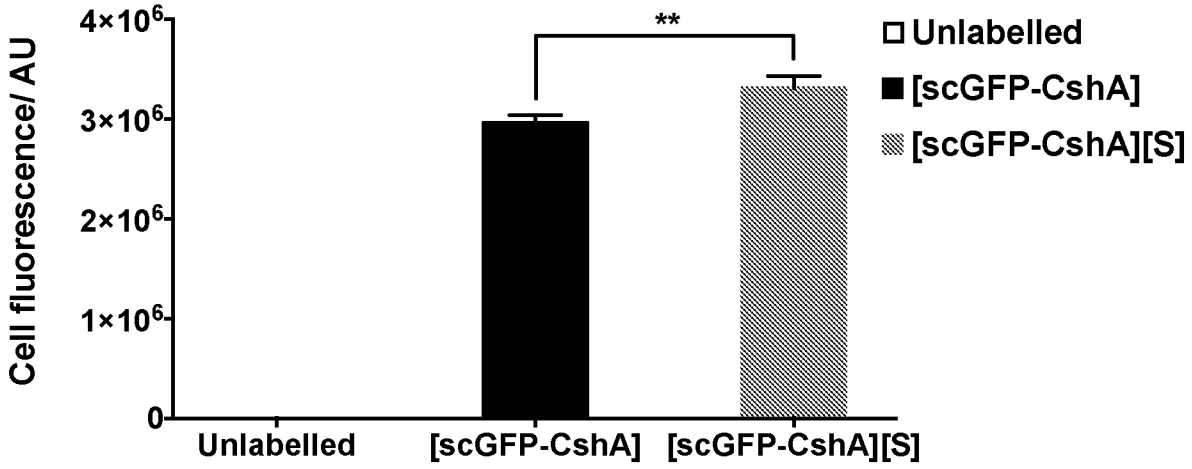

Figure 16

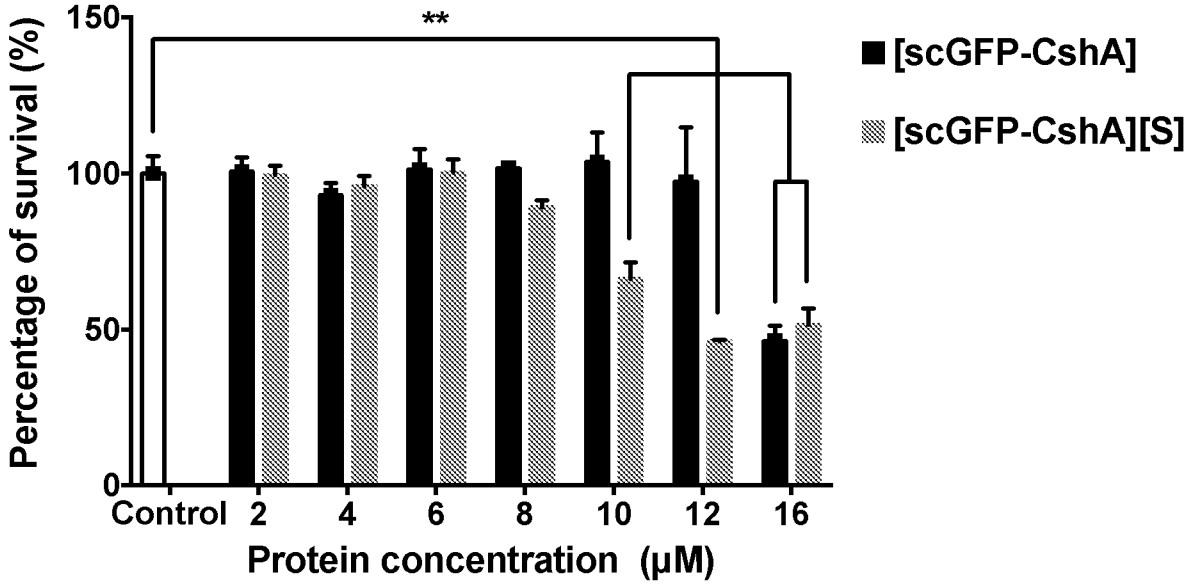

Figure 17

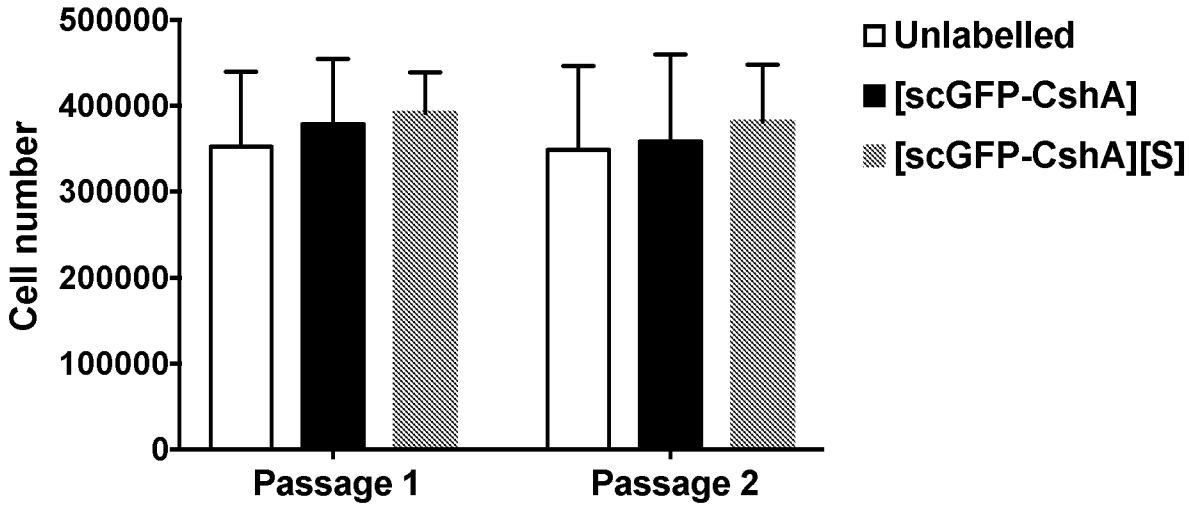

Figure 18

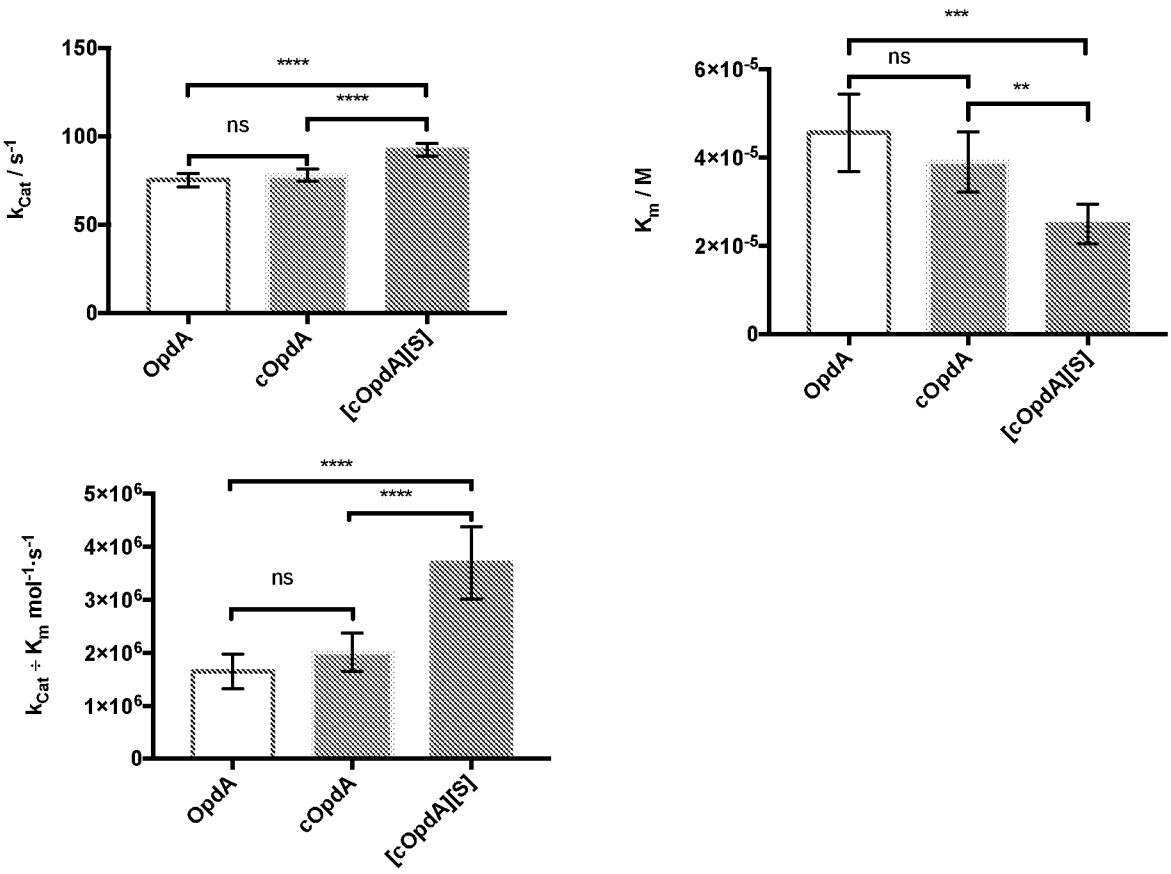

Figure 19

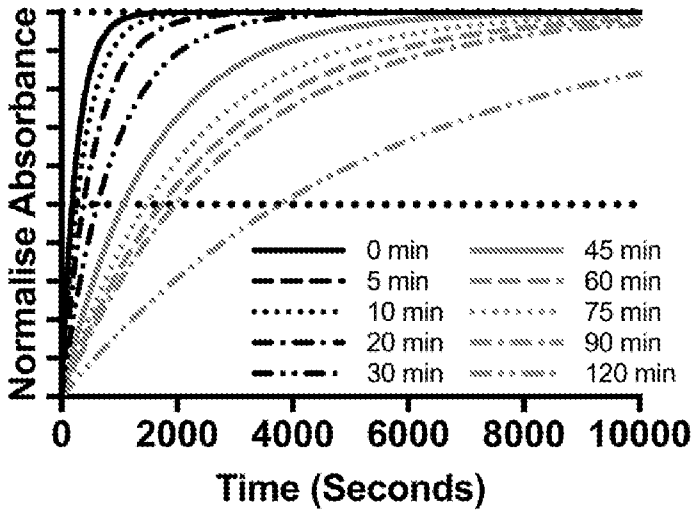

Figure 20

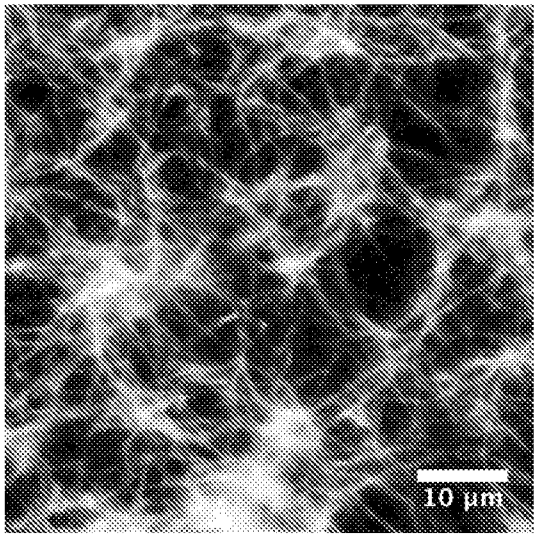

Figure 21

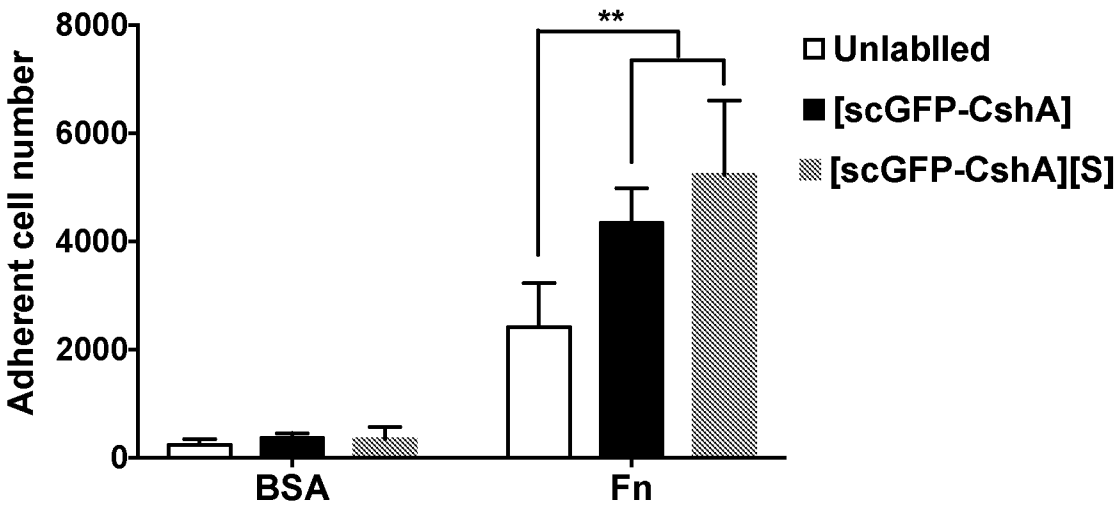

Figure 22

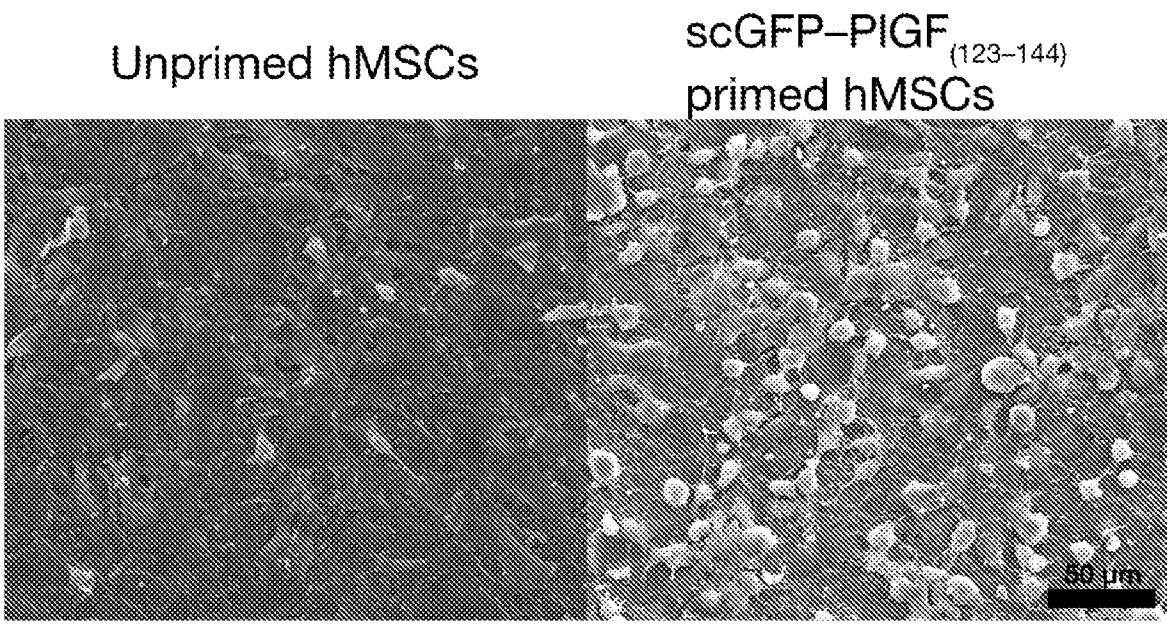

Figure 23

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2018/052534

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/00 C07K14/705 A61K38/00 A61K47/50
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)
C07K A61K

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, Sequence Search, BIOSIS, EMBASE, WPI Data, COMPENDEX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE Geneseq [Online] 14 August 2014 (2014-08-14), "Agrobacterium radiobacter OPDA protein, SEQ:3.", XP002786298, retrieved from EBI accession no. GSP:BBI93008 Database accession no. BBI93008 95.9% identity to present SEQ ID NO:11, 13, 40; abstract; sequence ----- -/--	42-46

[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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"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

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
7 November 2018	05/12/2018

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Kania, Thomas

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE Geneseq [Online] 26 March 2015 (2015-03-26), "Fusion protein sGFP-Tbx18-11R, SEQ 154.", XP002786299, retrieved from EBI accession no. GSP:BBU45831 Database accession no. BBU45831 92.8% identity to the supercharged GFP protein of present SEQ ID NO:15; abstract; sequence	42-46
X	DATABASE UniProt [Online] 23 October 2007 (2007-10-23), "Surface-associated protein CshA", XP002786300, Database accession no. A8AWJ3 89.4% identity to present SEQ ID NO:14, 16; abstract	42,43
X	JAMES P. K. ARMSTRONG ET AL: "Artificial membrane-binding proteins stimulate oxygenation of stem cells during engineering of large cartilage tissue", NATURE COMMUNICATIONS, vol. 6, no. 1, 17 June 2015 (2015-06-17), XP055521280, DOI: 10.1038/ncomms8405 cited in the application the whole document	1-4,6, 8-13,18, 20,21, 24, 26-28, 30,33,34
A	DE 10 2005 032055 A1 (FRAUNHOFER GES FORSCHUNG [DE]) 25 January 2007 (2007-01-25)	1-46
A	FUTAMI JUNICHIRO ET AL: "Exploiting protein cationization techniques in future drug development.", EXPERT OPINION ON DRUG DISCOVERY FEB 2007, vol. 2, no. 2, February 2007 (2007-02), pages 261-269, XP002786301, ISSN: 1746-0441	1-46
A	WO 2010/129023 A2 (HARVARD COLLEGE [US]; LIU DAVID R [US]; MCNAUGHTON BRIAN R [US]; CRONI) 11 November 2010 (2010-11-11)	1-46
A	US 9 259 398 B1 (KLEIN KYLE [US] ET AL) 16 February 2016 (2016-02-16)	1-46

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2018/052534

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		JP 2012525146 A	22-10-2012
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