SUMMARY

According to the present disclosure there is provided method for preparing a biologically active complex, said method comprising dissolving a mixture of a polypeptide element in powder form and oleic acid or a pharmaceutically acceptable salt thereof also in solid form, in an aqueous solvent comprising at least two salts, the first of which is sodium or potassium chloride and the second of which is disodium phosphate or mono-potassium phosphate, wherein, in particular, the method is carried out at moderate temperature.

As used herein, the expression ‘moderate temperature’ refers to temperatures of up to 50°C, for example from 0-50°C, for example from 10-40°C, and more particularly from 15-25°C, such as at ambient temperature. Such temperatures are generally below the ‘melt temperature’ at which the polypeptides become unfolded or denatured. However, the applicants have found that they are still able to form biologically active complexes under these salt conditions.

The applicants have found that active complex can be prepared which shows a clear dose-dependent response, by the simple dissolution method of the disclosure. Although the mixture may be warmed for example to temperatures of up to 50°C, such as up to 40°C to achieve rapid dissolution, there is no need to heat the solution extensively such as described in by boiling, provided only that a suitable salt balance is present in the aqueous solvent. Thus in a particular example, the method is carried out at ambient temperature.

Dissolution may be facilitated by agitation, for example by vortexing. If required, the solution may be filtered through a sterile filter at this stage. Suitable filters include polyethersulfone membranes (PES) or Minisart® NML Cellulose acetate membranes.

Any such agitation processes will be carried out for a period of time sufficient to ensure the dissolution of the elements in the salt solution. Although the precise timings may vary depending upon factors such as the particular nature of the polypeptide being used and the temperature at which the mixture is held, the timings will typically be quite short, for example no more than 10 minutes, for example from 1-5 minutes such as about 2 minutes.

In a particular example, the solvent further comprises a third salt which is mono-sodium or mono-potassium phosphate, and in particular is mono-potassium phosphate. Such mixtures are found in conventional PBS solutions.

This method is therefore easy to prepare in a variety of manufacturing and non-manufacturing environments.

The term “polypeptide” used herein includes proteins and peptides including long peptides.

Suitable “polypeptide elements” for use in the method of the disclosure include naturally-occurring proteins, in particular alpha-lactalbumin, lysozyme or other proteins having a membrane perturbing activity, recombinant proteins and in particular variants of said naturally-occurring proteins which lack intra-molecular bonds for example as a result of mutation of cysteine residues, or in particular, fragments of any of these proteins, in particular peptides of up to 50 amino acids.

The expression “variant” refers to proteins or polypeptides having a similar biological function but in which the amino acid sequence differs from the base sequence from which it is derived in that one or more amino acids within the sequence are substituted for other amino acids. Amino acid substitutions may be regarded as “conservative” where an amino acid is replaced with a different amino acid with broadly similar properties. Non-conservative substitutions are where amino acids are replaced with amino acids of a different type.

By “conservative substitution” is meant the substitution of an amino acid by another amino acid of the same class, in which the classes are defined as follows:

Class	Amino acid examples
Nonpolar:	A, V, L, I, P, M, F, W
Uncharged polar:	G, S, T, C, Y, N, Q
Acidic:	D, E
Basic:	K, R, H.

As is well known to those skilled in the art, altering the primary structure of a peptide by a conservative substitution may not significantly alter the activity of that peptide because the side-chain of the amino acid which is inserted into the sequence may be able to form similar bonds and contacts as the side chain of the amino acid which has been substituted out. This is so even when the substitution is in a region which is critical in determining the peptide’s conformation.

Non-conservative substitutions are possible provided that these do not interrupt the function of the DNA binding domain polypeptides.

Broadly speaking, fewer non-conservative substitutions will be possible without altering the biological activity of the polypeptides.

Determination of the effect of any substitution (and, indeed, of any amino acid deletion or insertion) is wholly within the routine capabilities of the skilled person, who can readily determine whether a variant polypeptide retains the fundamental properties and activity of the basic protein. For example, when determining whether a variant of the polypeptide falls within the scope of the disclosure, the skilled person will determine whether complexes

comprising the variant retain biological activity (e.g tumour cell death) of complexes formed with unfolded forms of the native protein and the polypeptide has at least 60%, preferably at least 70%, more preferably at least 80%, yet more preferably 90%, 95%, 96%, 97%, 98%, 99% or 100% of the native protein.

Variants of the polypeptide may comprise or consist essentially of an amino acid sequence with at least 70% identity, for example at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 96%, 97%, 98% or 99% identity to a native protein sequence such as an alphalactalbumin or lysozyme sequence.

The level of sequence identity is suitably determined using the BLASTP computer program with the native protein sequences as the base sequence. This means that native protein sequences form the sequence against which the percentage identity is determined. The BLAST software is publicly available at http://blast.ncbi.nlm.nih.gov/Blast.cgi (accessible on 10 May 2017).

In a particular example, the polypeptide element is a peptide that has no more than 50 amino acids, and in particular may have from 10-45 amino acids. Such complexes are easier to prepare and the starting materials are less costly. For instance, peptides may be prepared using conventional methods for the production of peptides. The complexes formed may be easier to handle and formulate for administration, due to the smaller molecular weight.

It is suitably derived from a naturally occurring protein or a variant thereof. Suitable proteins are those identified as being active in such complexes, such as alpha-lactalbumin, beta-lactoglobulin or lysozyme, but may be derived from any membrane perturbing proteins.

Membrane perturbing proteins are proteins which have the capability of interacting with the interface of cell membranes, in particular causing disruption such as tubulation of the cell membrane. Typically, the protein will become embedded in the cell membrane. Examples of such proteins include coat complexes such as COPI, COPII (such as SAR 1), HOPS/CORVET, SEA (Seh1-associated), and clathrin complexes BAR domain proteins such as endophilins, and the ESCRT complex, including Snf7 domain subunits.

In particular, the peptide is derived from the alpha-helical domain of a naturally occurring protein as described above. The alpha-helical domain of said proteins would be well understood in the art or may be determined using conventional methods.

Where the alpha-helical domain contains a cysteine residue, these may, in some embodiments, be modified to a different amino acid residue, such as an alanine residue, in order to avoid inter-molecular disulphide bonds.

In a particular example, the peptide is a fragment of alpha-lactalbumin and specifically a fragment of the alpha domain of alpha-lactalbumin. In a particular example, the peptide comprises amino acids of the Alpha 1 (residues 1-40) or Alpha 2 (residues 81-123) of human alpha-lactalbumin, or analogous regions of other alphalactalbumins such as bovine alpha-lactalbumin.

The peptide suitably contains no elements that give rise to folding and therefore suitably lacks amino acids that give rise to intramolecular bonding such as cysteine residues. In particular, where the peptide is derived from a naturally occurring protein, any cysteine residues are replaced by other amino acids such as alanine.

Thus in a particular example, the complex comprises amino acids of the Alpha 1 (residues 1-40) or Alpha 2 (residues 81-123) of human alpha-lactalbumin wherein the cysteines are replaced with other amino acids such as alanine, to prevent any intra-molecular bonding.

Thus the peptide may be of SEQ ID NO 1 or SEQ ID NO 2

KQFTK**X**ELSQLLKDIDGYGGIALPELI**X**TMFHTSGYDTQA (SEQ ID NO 1)

LDDDITDDIM**X**AKKILDIKGIDYWLAHKAL**X**TEKLEQWL**X**EKL (SEQ ID NO 2)
where X is an amino acid residue other than cysteine.

A particular example of such sequences are those of SEQ ID NO 3 or SEQ ID NO 4.

KQFTK**A**ELSQLLKDIDGYGGIALPELI**A**TMFHTSGYDTQA (SEQ ID NO 3)

LDDDITDDIM**A**AKKILDIKGIDYWLAHKAL**A**TEKLEQWL**A**EKL (SEQ ID NO 4).

In some cases, the peptides of SEQ ID NO 1 may be truncated, for example by omitting the terminal alanine residue, resulting in a peptide of SEQ ID No 6, of which SEQ ID NO 7 is a specific example.

KQFTK**X**ELSQLLKDIDGYGGIALPELI**X**TMFHTSGYDTQ (SEQ ID NO 6)

KQFTK**A**ELSQLLKDIDGYGGIALPELI**A**TMFHTSGYDTQ (SEQ ID NO 7)

Such peptides are novel and form a further example of the disclosure, together with biologically active complexes comprising them.

In one aspect, there is provided a biologically active complex comprising a peptide of SEQ ID NO 6 and oleic acid or a salt thereof.

In another aspect, there is provided a peptide of SEQ ID NO: 6.

Other peptides may also be used in the complex and the suitability may be tested by determining whether complexes with a fatty acid salt are active, for instance in killing cells using methods as described hereinafter

In another example, the peptide is derived from a COPII family protein such as SAR1. A particular example of such a peptide is a peptide of SEQ ID NO 5

MAGWDIFGWF RDVLASLGLW NKH (SEQ ID NO 5).

In another example, the polypeptide element is a naturally-occurring protein or a synthetic form thereof, in particular an alpha-lactalbumin, such as human, bovine, sheep, camel or goat alpha-lactalbumin. In particular, the protein is bovine lactalbumin.

As used herein, the term “biologically active” means that the complex has a biological activity, which is different from - or stronger than the individual components. In particular, the complex is able to induce cell death in particular selectively in tumour cells and/or has a bactericidal or antiviral effect not seen with the native protein including for example monomeric α-lactalbumin forms, although other therapeutic effects may be available.

In particular oleic acid used in the method of the disclosure is C18:1 oleic acid of formula $CH_3(CH_2)_7CH=CH(CH_2)_7COOH$ or $CH_3(CH_2)_7CH=CH(CH_2)_7COO^-$.

In a particular example, a pharmaceutically acceptable salt of oleic acid is used in the process. Suitable pharmaceutically acceptable salts would be understood in the art.

Use of a salt, and in particular a water-soluble salt of the oleic acid, fatty acid or lipid means that the preparation method is facilitated since aqueous solutions may be formed for instance for application to ion exchange columns and the like. Suitable water-soluble salts are alkali or alkaline-earth metal salts such as sodium or potassium salts.

Furthermore, it has been found that salts and in particular oleate salts such as sodium oleate appear to have some inherent tumoricidal effect. Therefore the inclusion of this in the complex may give rise to activity increases.

In a particular example, the first salt used in the method of the disclosure is sodium chloride.

In another particular example, the second salt used in the method of the disclosure is disodium phosphate.

In another particular example, the third salt used in the method of the disclosure is mono-potassium phosphate.

The ratio of first salt : second salt used in the method of the disclosure is suitably from 8:1 to 1:1, for example from 5:1 to 2:1 and in particular from 4:1 to 3.5:1. Where present the ratio of first salt: third salt is from 20:1 to 5:1, for example from 15:1 to 10:1 such as from 12.5:1 to 11.5:1.

In a particular example the ratio of first to second to third salt is 13-12:4-3:1.

The ratio of oleic acid or oleate: peptide mixed in the method of the disclosure is suitably in the range of from 20:1 to 1 to 1, but preferably an excess of oleate is present, for instance in a ratio of oleate:peptide of about 5:1. The mixing can be carried out at a temperature of from 0-50°C, conveniently at ambient temperature and pressure.

If required, the product of the process of the disclosure may solidified for example by lyophilisation, for storage or for formulation purposes. Thereafter, it may be reconstituted using in particular, sterile water, for use. Such procedures may be particularly suitable where the polypeptide is a peptide rather than a protein. The applicants have found that proteins may revert to the natural folding state when subjected to procedures such as lyophilisation.

The problem may be alleviated by stabilising the polypeptide in the unfolded state, for example by lowering the pH of the solution, for instance to a value of 4 or less, or adding calcium chelators such as EDTA to the solvent during the preparation method. In a second example, the disclosure provides a complex obtainable by the method of the first aspect.

Thus, complexes of the second example of the disclosure may be formulated into useful pharmaceutical compositions by combining them with pharmaceutically acceptable carriers in the conventional manner. Such compositions form a third example of the disclosure. In another aspect, there is provided a pharmaceutical composition comprising a biologically active complex as described herein, and a pharmaceutically acceptable carrier.

The compositions in accordance with the third example of the disclosure are suitably pharmaceutical compositions in a form suitable for topical use, for example as creams, ointments, gels, or aqueous or oily solutions or suspensions. These may include the commonly known carriers, fillers and/or expedients, which are pharmaceutically acceptable.

Topical solutions or creams suitably contain an emulsifying agent for the protein complex together with a diluent or cream base.

The daily dose of the complex varies and is dependant on the patient, the nature of the condition being treated etc. in accordance with normal clinical practice. As a general rule from 2 to 200 mg/dose of the biologically active complex is used for each administration.

In a further example of the disclosure, there is provided a method for treating cancer which comprises administering to a patient in need thereof, a biologically active complex as described above.

In another aspect, there is provided a biologically active complex as described herein, or a pharmaceutical composition as described herein, for use in the treatment or prevention of cancer or a viral or bacterial infection.

In particular, the complex may be used to treat cancers such as human skin papillomas, human bladder cancer and glioblastomas. In the latter case, administration may be by infusion as is known in the art.

The disclosure further provides the biologically active complex as defined above for use in therapy, in particular in the treatment of cancer.

The complex may also be of use in the prevention of cancer, in particular gastrointestinal cancer as described for example in WO2014/023976. In this case, the complex may be combined with a foodstuff, such as a dairy product such as yoghurt for use as a nutraceutical. Compositions of this type form a further example of the disclosure. In another aspect, there is provided a nutraceutical composition comprising a biologically active complex as described herein.

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. Moreover 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.

Preferred features of each aspect of the invention may be as described in connection with any of the other aspects. Within the scope of this application it is expressly intended that the various aspects, embodiments, examples and alternatives set out in the preceding paragraphs, in the claims and/or in the following description and drawings, and in particular the individual features thereof,, may be taken independently or in any combination. That is, all embodiments and/or features of any embodiment can be combined in any way and/or combinations, unless such features are incompatible.

BRIEF DESCRIPTION OF THE DRAWINGS

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

Figure 1 shows the results of ATP Lite, PrestoBlue and Trypan Blue studies obtained using a range of biologically active complexes on tumour cells as described below;

Figure 2 shows a comparison of similar results obtained with and without filtration of the solutions;

Figure 3 shows (A) a schematic and photograph of the preparation of a complex comprising bovine alpha-lactalbumin, and (B) results of ATP Lite, PrestoBlue and Trypan Blue studies of tumour cells to which the resultant solution was administered.

EXAMPLE 1

<u>Production of biologically acceptable complexes</u>

A range of biologically active complexes were prepared using a peptide of SEQ ID NO 7

Ac-KQFTKAELSQLLKDIDGYGGIALPELIATMFHTSGYDTQ-OH (SEQ ID NO 7)

Claims

1. A biologically active complex comprising a peptide of SEQ ID NO 6 and oleic acid or a salt thereof.

2. A biologically active complex according to claim 1 wherein the peptide is of SEQ ID NO 7.

3. A peptide of SEQ ID NO 6.

4. A peptide according to claim 3 which is of SEQ ID NO 7.

5. A pharmaceutical composition comprising a biologically active complex according to claims 1 or 2, and a pharmaceutically acceptable carrier.

6. A biologically active complex according to claims 1 or 2, or a pharmaceutical composition according to claim 5, for use in the treatment or prevention of cancer or a viral or bacterial infection.

7. A nutraceutical composition comprising a biologically active complex according to claim 1 or 2.