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(54) Title: PHARMACEUTICALLY ACTIVE PROTEIN CRYSTALS GROWN IN-SITU WITHIN A HYDROGEL

(57) Abstract: The present invention provides a process to manufacture a composition comprising a hydrogel comprising pharmaceutically active protein crystals grown in-situ within the hydrogel, wherein the pharmaceutically active protein crystals grown in-situ have an average size of less than 20 microns, which comprises: a. Sequentially blending a pharmaceutically active protein solution in their appropriate buffer composition by using a value of supersaturation in the metastable zone width (MSZW) or nearby the supersolubility curve, with a precipitant solution, wherein acetone is absent, and a gelator solution, b. Inducing the nucleation by lowering the temperature, and c. Storing the resulting mixture at a constant temperature, preferably at room temperature at 1 atm.

Pharmaceutically active protein crystals grown *in-situ* within a hydrogel**FIELD OF THE INVENTION**

The present invention refers to the field of protein crystallization, in particular it is directed to protein crystals grown *in situ* within a hydrogel having an average size of 20 or less microns.

BACKGROUND OF THE INVENTION

The field of protein crystallization is of crucial importance to unveil the secrets of biological systems at the molecular level. It has an immediate impact in structural proteomic/genomic projects as well as in rational drug design. For this reason, growing crystals of adequate size and quality for applications such as X-ray diffraction or therapeutic purposes is often the major bottleneck. Many different factors have an influence on the whole process of protein crystallization and therefore a multitude of methods, strategies and techniques have been developed to attain success. In most cases, the optimal strategy to obtain crystals of a particular protein is found serendipitously. One emerging strategy in this field employs the use of hydrogels as media or carriers for the growth of protein crystals. It has been demonstrated that the use of conventional macromolecular hydrogels such as agarose, polyacrylamide, silica and sephadex has a direct impact on the formation of protein crystals and their quality. Indeed, crystals of exceptional size and quality are obtained within hydrogels when compared with other traditional crystallization techniques. This can be explained by (i) the physical properties of the hydrogel which eliminates sedimentation, convection current and acts as impurity filter media, and (ii) their molecular influence, as hydrogel fibers can interact directly with protein molecules, having a final composite formed by polymeric fibers of agarose, silica, and PEG-based hydrogels, incorporated within the crystal lattices of protein crystals. This incorporation occurs during the growth process, thus influencing crystal polymorphism, enantiomorphism, habits and stabilities.

Compositions comprising a hydrogel which in turn comprises pharmaceutically active protein crystals grown *in-situ*, such as insulin, within the hydrogel, provide a number of significant advantages to the crystals such as greater stability and controlled liberation. However, all of the methods performed so far in which hydrogels have been used as media or carriers for the growth of protein crystals within the hydrogel have resulted in a wide range of crystal sizes, see figure 1. Such wide range of sizes might be useful to carry out an X-Ray diffraction characterization but useless in therapeutic treatment. For therapeutic treatment a narrow range of crystal sizes with an average size of 20 or less microns, preferably of 10 or less microns is needed.

Therefore, there is still a need to provide for a methodology that allows the production of protein crystals grown *in situ* within a hydrogel having an average size of 20 or less microns, preferably of 10 or less microns, so they can be used directly in therapeutic treatment.

5 BRIEF DESCRIPTION OF THE INVENTION

The present invention solves the above mentioned problem by providing a process to manufacture a composition comprising a hydrogel comprising pharmaceutically active protein crystals grown *in-situ* within the hydrogel, wherein the pharmaceutically active protein crystals grown *in-situ* have an average size of 20 or less microns, preferably of 19 or less microns, preferably of 18 or less microns, preferably of 17 or less microns, preferably of 16 or less microns, preferably of 15 or less microns, preferably of 14 or less microns, preferably of 13 or less microns, preferably of 12 or less microns, preferably of 11 or less microns, preferably of 10 or less microns, preferably of 9 or less microns, preferably of 8 or less microns, preferably of 7 or less microns, preferably of 6 or less microns, preferably of 5 or less microns measured by scanning electronic microscopy, which comprises:

- a. Sequentially blending a pharmaceutically active protein solution in their appropriate buffer composition by using a value of supersaturation in the metastable zone width (MSZW) or nearby the supersolubility curve, with a precipitant solution, wherein acetone is absent, and a gelator solution,
- b. Inducing the nucleation by lowering the temperature, and
- c. Storing the resulting mixture at a constant temperature, preferably at room temperature at 101325 Pa (1 atm).

The aforesaid methodology provides for a pharmaceutically acceptable composition comprising a hydrogel which in turn comprises pharmaceutically active protein crystals grown *in-situ* within the hydrogel,

- wherein the hydrogel is a viscoelastic solid-like material comprising an elastic cross-linked network and water, wherein water is the major component;
- wherein the pharmaceutically active protein crystals are composite materials of the protein in crystalline state having an average size of 20 or less microns, preferably of 19 or less microns, preferably of 18 or less microns, preferably of 17 or less microns, preferably of 16 or less microns, preferably of 15 or less microns, preferably of 14 or less microns, preferably of 13 or less microns, preferably of 12 or less microns, preferably of 11 or less microns, preferably of 10 or less microns, preferably of 9 or less microns,

preferably of 8 or less microns, preferably of 7 or less microns, preferably of 6 or less microns, preferably of 5 or less microns, measured by scanning electronic microscopy;

- wherein the hydrogel can be either macromolecular or supramolecular; and
- wherein acetone is absent from the composition.

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BRIEF DESCRIPTION OF THE FIGURES

Fig. 1. Photos of crystals obtained in the publication CrystEngComm, 2015, 17, 8072-8078, with maximum and minimum sizes measured.

10 **Fig. 2.** Photos of crystals obtained according to examples 1 and 2 with the maximum and minimum sizes measured.

Fig. 3. Phase diagram for the crystallization of macromolecules. The solubility diagram is divided sharply into a region of undersaturation and a region of supersaturation by the line denoting maximum solubility at specific concentrations of a precipitant, which may be salt or a polymer. **The line represents the equilibrium between the existence of the solid phase and the free-molecule phase.** The region of supersaturation is further divided in a more uncertain way into the metastable and labile regions. In the metastable region nuclei will develop into crystals, but no nucleation will occur. In the labile region both might be expected to occur. The final region, at very high supersaturation, is denoted the precipitation region, where this result might be most probable. Crystals can only be grown from a supersaturated solution, and
15
20 creating such a solution supersaturated in the protein of interest is the immediate objective in growing protein crystals.

Fig. 4. Schematic illustration of a protein crystallization phase diagram. Adjustable parameters include precipitant or additive concentration, pH and temperature. The four major crystallisation methods are represented: (i) microbatch, (ii) vapor diffusion, (iii) dialysis and (iv) FID (free interface diffusion). Each involves a different route to reach the nucleation and metastable zones, assuming the adjustable parameter is precipitant concentration. The filled black circles represent the starting conditions. Two alternative starting points are shown for FID and dialysis because the undersaturated protein solution can contain either protein alone or protein mixed with a low concentration of the precipitating agents. The solubility is defined as the concentration of protein in the solute that is in equilibrium with crystals. The supersolubility curve is defined as the line separating conditions under which spontaneous nucleation (or phase separation or precipitation) occurs from those under which the crystallisation solution remains clear if left undisturbed.
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30

35 **Fig. 5.** Preliminary stability tests of crystalline doses of insulin in solution and in agarose and di-alanine hydrogels.

Fig. 6. Schematic representation of protocol 1 to study the pharmacokinetic behaviour of the different insulin formulations.

Fig. 7. Graphic representation of the evolution of glycaemia at two different concentrations to establish initial insulin doses.

Fig. 8. Results of the pharmacokinetic behaviour of the reference insulin, insulin dialanine and insulin agarose at 1x doses.

Fig. 9. Schematic representation of protocol 2 to study the pharmacokinetic behaviour of the different insulin formulations.

Fig. 10. A long term study of the effect of insulin dialanine (10x), single dose, compared to the vehicle.

Fig. 11. Schematic representation of protocol 3 to study the pharmacokinetic behaviour of the different insulin formulations.

Fig. 12. Results of the pharmacokinetic behaviour of the reference insulin and insulin dialanine at 1x (A), 5x (B) and 10x (C) doses.

Fig. 13. Results of the pharmacokinetic behaviour of the reference insulin and insulin dialanine kept at 50 °C at 5x dose. Results are shown as mg/dl of glucose in blood (left) and % of decrease of glucose in blood (right).

Fig. 14. Results of the pharmacokinetic behaviour of the reference insulin and insulin dialanine kept at 50 °C at 10x dose. Results are shown as mg/dl of glucose in blood (left) and % of decrease of glucose in blood (right).

DETAILED DESCRIPTION OF THE INVENTION

Definitions

For the purpose of the present invention, the following definitions are included below:

- The term "*comprising*" it is meant including, but not limited to, whatever follows the word "*comprising*". Thus, use of the term "*comprising*" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present.

- By "*consisting of*" is meant including, and limited to, whatever follows the phrase "consisting of". Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present.
- As used herein "solubility" is defined as the concentration of protein in the solution that is in equilibrium with the protein in crystalline state.
- As used herein "supersolubility curve" is defined as the line separating conditions under which spontaneous nucleation (or phase separation or precipitation) occurs from those under which the crystallisation solution remains clear if left undisturbed.
- As used herein "labile region" is understood as the supersaturated region in which both nucleation and growth might be expected to occur.
- As used herein "precipitation region" is understood as the zone of the phase diagram above the labile zone in which only amorphous (non-crystalline material) is obtained.
- As used herein "metastability zone" is understood as the region of the phase diagram where nuclei will develop into crystals, but no nucleation will occur.
- As used herein "region of sub-saturation or under-saturation" is understood as zone of the phase diagram in which the protein concentration is lower than the solubility.
- As used herein "precipitant" is understood as the compound or mixture of compounds that provokes the precipitation of the protein in solution when the concentrations are above the solubility curve.
- As used herein "hydrogel" is a viscoelastic solid-like material comprising an elastic cross-linked network and water, wherein water is the major component.
- As used herein "gelling agent" is a compound capable of forming a hydrogel.
- As used herein "average size" is understood as sum of the lengths of all the measured crystals divided by the number of measured lengths.
- As used herein "crystals-lengths" are defined as the two major dimensions of the rhombohedral insulin-crystal.
- As used herein "Di-Phe" is the dipeptide N-(9-Fluorenylmethoxycarbonyl)-L-phenylalanine-L-phenylalanine, terminated by a free carboxyl group at their C-terminus end.
- As used herein "Di-Ala" is the dipeptide N-(9-Fluorenylmethoxycarbonyl)-L-alanine-L-alanine.
- As used herein "Fmoc-AA-OH" is either the dipeptide N-(9-Fluorenylmethoxycarbonyl)-L-alanine-L-alanine, N-(9-Fluorenylmethoxycarbonyl)-D-alanine-L-alanine, N-(9-Fluorenylmethoxycarbonyl)-L-alanine-D-alanine or N-(9-Fluorenylmethoxycarbonyl)-D-alanine-D-alanine.
- As used herein "Fmoc-CF-OH" refers to the dipeptide N-(9-Fluorenylmethoxycarbonyl)-L-cysteine-L-phenylalanine, wherein cysteine and phenylalanine can be independently of each other in the L or D configuration;

- As used herein "Fmoc-MF-OH" refers to the dipeptide N-(9-Fluorenylmethoxycarbonyl)-L-methionine-L-phenylalanine, wherein methionine and phenylalanine can be independently of each other in the L or D configuration;
- As used herein "Fmoc-FF-OH" refers to the dipeptide N-(9-Fluorenylmethoxycarbonyl)-L-phenylalanine -L-phenylalanine, wherein each phenylalanine can be independently of each other in the L or D configuration.

Description

The present invention provides a new methodology that allows the production of protein crystals grown *in situ* within a hydrogel having an average size of 20 or less microns, preferably of 10 or less microns, so they can be used directly in therapeutic treatment. The described methodology is based on batch crystallization method in gelled media and is therefore scalable.

In particular, in the methodology of the present invention a solution containing the active ingredient, the protein, is mixed with a solution comprising a precipitant and a solution comprising a gelling agent at a temperature that keeps the protein in a sub-saturation or low supersaturation state, in particular in the metastability zone (see figure 3). The solution is then brought to a supersaturation state, such as the labile region or the precipitation region, to induce nucleation by means of thermal shock, i.e by cooling, and is kept at said temperature for the required time before bringing it to a metastability state, in which crystal growth is allowed without new nucleation events occurring.

This principle is explained in figure 3 wherein a solubility diagram is shown divided sharply into a region of sub-saturation or under-saturation and a region of supersaturation by the line denoting maximum solubility at specific concentrations of a precipitant, which may be salt or a polymer. This line represents the equilibrium between the existence of the solid phase and the free-molecule phase in solution. The region of supersaturation is further divided into the metastable and labile regions. In the metastable region nuclei will develop into crystals, but no nucleation will occur. In the labile region both might be expected to occur (nucleation and crystal growth). The final region, at very high supersaturation, is denoted the precipitation region. Crystals can only be grown from a supersaturated solution, and creating such a solution supersaturated in the protein of interest is the immediate objective in growing protein crystals.

To understand the difference between the methodology herein presented and the one described previously, the fundamental aspects of both will be described.

According to the methodology previously described (see CrystEngComm, 2015, **17**, 8072-8078), Hydrogels were prepared in MilliQ water by heating in a closed vial in the case of hydrogel 1 and 2 while hydrogels of Di-Phe or Di-Ala were prepared by dissolving the peptide in

5 μL DMSO to a final concentration of 100 mg/mL followed by the addition of 100 μL of MilliQ water. The excess of DMSO in the formed hydrogels was then removed by the addition of an excess of MilliQ water in each Eppendorf for 12 hours. This process was repeated several times for a week.

5 Then, a counter-diffusion technique with two layers configuration (2L) was used to set-up crystallization experiments in Eppendorf tubes. Two different set-ups were tested. In the first case the protein was allowed to diffuse within a pre-set hydrogel column (50 μL) for one week while in the second case protein solutions were directly mixed with the hydrogel precursor to a final volume of 50 μL , and let it gel. Then, 50 μL of the precipitant solution were added on top of
10 the hydrogel plus protein layer to start the crystallization experiment.

The fundamental difference between the methodology previously described as detailed above and the methodology of the present invention is the crystallization technique and, accordingly, the product derived from said crystallization technique, the crystals.

15 In the gel counter-diffusion technique as illustrated in methodologies previously described, the protein (active ingredient) must be first contained in a gel regardless of the method used for its inclusion, and therefore the protein is contained in the gel in absence of a precipitating agent. To cause precipitation/crystallization, a precipitating agent is later diffused, the diffusion of which through the protein chamber gradually increases supersaturation giving rise to precipitation events which may or may not be in the form of crystals. The combination of
20 precipitation with the continuous advancement of the precipitating agent allows obtaining crystals having a wide range of sizes. Crystals further away from the point at which the precipitating agent is incorporated are expected to have a large size, this being an expected and desired result as described in many publications. It is therefore evident that the dynamic character of this technique does not allow controlling nucleation density or final crystal size and
25 generates a highly heterogeneous mixture of crystal sizes. For example and for the particular case of insulin, in the article published in Cryst. Growth Des. 2014, **14**, 3239–3248, PEG hydrogels are used to obtain large insulin crystals (more than 50 microns and exceeding 200 microns, see Figure 7 of the article). In CrystEngComm, 2015, **17**, 8072-8078, insulin crystals obtained using peptide hydrogels and agarose are described as having a small size, although in
30 this case the size varies greatly in the range of 30 to 100 microns, due to the counter-diffusion technique used for obtaining them (see figure 1) and are therefore useless in therapy.

In contrast, according to the methodology of the present invention the crystallization process is carried out using the batch method. In this sense and by definition, all the components (active ingredient {protein}, mixture of precipitant and gelling agent) are sequentially mixed, and the
35 system is placed in a supersaturation value in the metastability zone width, which allows controlling nucleation density and therefore final crystal size using thermal control to induce

nucleation and crystalline growth. The results are clearly illustrated in figure 2. In addition, as illustrated in the examples, the hydrogel-grown insulin crystals are particularly stable and are capable of providing a sustained hypoglycemic effect.

Therefore, a first aspect of the invention refers to a process to manufacture a composition comprising a hydrogel comprising pharmaceutically active protein crystals grown *in-situ* within the hydrogel, wherein the pharmaceutically active protein crystals grown *in-situ* have an average size of 20 or less microns, preferably of 19 or less microns, preferably of 18 or less microns, preferably of 17 or less microns, preferably of 16 or less microns, preferably of 15 or less microns, preferably of 14 or less microns, preferably of 13 or less microns, preferably of 12 or less microns, preferably of 11 or less microns, preferably of 10 or less microns, preferably of 9 or less microns, preferably of 8 or less microns, preferably of 7 or less microns, preferably of 6 or less microns, preferably of 5 or less microns, which comprises:

a. Sequentially blending a pharmaceutically active protein solution in an appropriate buffer composition by using a value of supersaturation in the metastable zone width (MSZW) or nearby the supersolubility curve, with a precipitant solution, wherein acetone is absent, and a gelator solution,

b. Inducing the nucleation by lowering the temperature, and

c. Storing the resulting mix at a constant temperature, preferably at room temperature at 1 atm.

In a preferred embodiment of the first aspect of the invention, the protein active solution is a solution comprising insulin, the gelator solution is the Fmoc-AA-OH hydrogel and the induction of the nucleation is performed by placing the product resultant from step a) at approximately 4°C, preferably for approximately 30 minutes. More preferably, the protein active solution is a solution comprising insulin, the precipitant solution is HCl, ZnCl₂ and sodium citrate, the gelator solution is the Fmoc-AA-OH hydrogel and the induction of the nucleation is performed by placing the product resultant from step a) at approximately 4°C, preferably for approximately 30 minutes. Preferably, the concentration of HCl is between 5 and 20 mM, the concentration of ZnCl₂ is approx. 5 mM and the concentration of sodium citrate is between 15 and 50 mM.

In another preferred embodiment of the first aspect of the invention, the protein active solution is an insulin solution, the gelator solution is agarose and the induction of the nucleation is carried out by allowing the product resultant from step a) to reach room temperature or actively cooling it to room temperature. More preferably, the protein active solution is an insulin solution, the precipitant solution is HCl, ZnCl₂ and sodium citrate, the gelator solution is agarose and the induction of the nucleation is carried out by allowing the product resultant from step a) to reach room temperature or actively cooling it to room temperature. Preferably, the concentration of

HCl is between 5 and 20 mM, the concentration of ZnCl₂ is approx. 5 mM and the concentration of sodium citrate is between 15 and 50 mM.

A second aspect of the invention refers to a composition comprising a hydrogel comprising pharmaceutically active protein crystals grown *in-situ* within the hydrogel, obtained or obtainable by the process of the first aspect of the invention.

A third aspect of the invention refers to composition comprising a hydrogel which in turn comprises pharmaceutically active protein crystals grown *in-situ* within the hydrogel,

- wherein the hydrogel is a viscoelastic solid-like material comprising an elastic cross-linked network and water, wherein water is the major component;
- wherein the pharmaceutically active protein crystals are composite materials of the protein in crystalline state having an average size of 20 or less microns, preferably of 19 or less microns, preferably of 18 or less microns, preferably of 17 or less microns, preferably of 16 or less microns, preferably of 15 or less microns, preferably of 14 or less microns, preferably of 13 or less microns, preferably of 12 or less microns, preferably of 11 or less microns, preferably of 10 or less microns, preferably of 9 or less microns, preferably of 8 or less microns, preferably of 7 or less microns, preferably of 6 or less microns, preferably of 5 or less microns, measured by scanning electronic microscopy;
- wherein the hydrogel can be either macromolecular or supramolecular; and
- wherein acetone is absent from the composition.

In a preferred embodiment of the third aspect of the invention, the hydrogel is macromolecular and comprises or consists of a compound selected from the group consisting of agarose, gelatin, carrageenan, Poly(ethylene glycol) (PEG). Preferably, the agarose gel comprises or consists of polysaccharides of agarobiose units, wherein agarobiose is a disaccharide formed by the union of D-galactose and 3,6-anhydro-L-galactose. Also preferably, the PEG hydrogel is composed of poly(ethylene glycol) monomethyl ether monomethacrylate (PEGMA) of average molecular weight (MW) 1100 Da cross-linked with poly(ethylene glycol) dimethacrylate (PEGDMA) of MW 1200 Da.

In another preferred embodiment of the third aspect of the invention, the hydrogel is a supramolecular peptide-based hydrogel. Preferably, the supramolecular peptide-based hydrogel is a cysteine-based peptide, more preferably: N, N'-di(benzoyl)-L or D-cysteine-diamide, or consists of peptides comprising an amino-acid chain of four or less amino-acids, preferably of two amino-acids, di-peptides. Preferably, the dipeptides are compounds selected from the group consisting of Fmoc-CF-OH, Fmoc-MF-OH, Fmoc-FF-OH and Fmoc-AA-OH. More preferably:

- the Fmoc-CF-OH hydrogel is based on the dipeptide N-(9-Fluorenylmethoxycarbonyl)-L-cysteine-L-phenylalanine;
- the Fmoc-MF-OH hydrogel is based on the dipeptide N-(9-Fluorenylmethoxycarbonyl)-L-methionine-L-phenylalanine;
- 5 - the Fmoc-FF-OH hydrogel is based on the dipeptide N-(9-Fluorenylmethoxycarbonyl)-L-phenylalanine -L-phenylalanine; and
- the Fmoc-AA-OH hydrogel is based on the dipeptide N-(9-Fluorenylmethoxycarbonyl)-L-alanine -L-alanine.

In another preferred embodiment of the third aspect of the invention or of any of its preferred
10 embodiments, the pharmaceutically active protein crystals are selected from the list consisting
of any of the following compounds: insulin, lysozyme, albumins, phosphatase alkaline, glucose
isomerase, growth hormone, somatotropin, factor VIII, factor IX, antithrombin III,
immunoglobulins, erythropoietin, interferons, papain, trypsin, hyaluronan-degrading enzymes,
15 collagenase, streptokinase, glucagon, thyrotropin, secretin, humanized chimeric mAB
(monoclonal antibodies), vaccines, HIV antigens and hepatitis C antigens. Preferably, the
pharmaceutically active protein crystals are insulin crystals. More preferably, the
pharmaceutically active protein crystals are insulin crystals and the hydrogel is a peptide-based
hydrogel formed by Fmoc-AA-OH or an agarose gel comprising polysaccharides of agarobiose
units. More preferably, the pharmaceutically active protein crystals are insulin crystals and the
20 hydrogel is formed by PEG with an average MW 1100 Da.

In a fourth aspect of the invention, the composition of any the second or third aspect of the
invention is a pharmaceutical composition.

In a fifth aspect of the invention, the composition of any the second or third or fourth aspect of
the invention is used in therapy.

25 In a sixth aspect of the invention, the composition of any the second or third or fourth aspect of
the invention is used in the controlled liberation of the pharmaceutically active protein crystals.

In a seventh aspect of the invention, the pharmaceutically active protein crystals of the
composition of the second or third aspects of the invention are insulin crystals and the
composition is used in the treatment of diabetes, in particular of type I and/or II diabetes. More
30 preferably, the pharmaceutically active protein crystals are insulin crystals and the hydrogel is a
peptide-based hydrogel formed by Fmoc-AA-OH or an agarose gel comprising polysaccharides
of agarobiose units and the composition is used in the treatment of diabetes, in particular of
type I and/or II diabetes. More preferably, the pharmaceutically active protein crystals are insulin
crystals and the hydrogel is formed by PEG with an average MW 1100 Da and the composition
35 is used in the treatment of diabetes, in particular of type I and/or II diabetes.

The invention has been described broadly and generically herein. Each of the narrower species and sub-generic groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

Other embodiments are within the following claims and non-limiting examples. In addition, where features or aspects of the invention are described in terms of groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the group.

EXAMPLES

Example 1. Insulin protein in agarose gel and DMSO

Insulin protein in agarose gel and DMSO was prepared by sequentially dissolving the insulin to a final concentration of 8 mg/mL, in 6 mM HCl, 5 mM ZnCl₂, 16 mM Sodium citrate pH 7.0, 15% DMSO and 0.04% agarose. The resulting preparation was stored at 20°C. The agarose hydrogel was prepared earlier by dissolving 0.08 grams of disaccharide in 10 mL of water at a final concentration of 0.8% and heating to boiling in order to completely dissolve it and kept at 45°C until it was mixed with the rest of the components.

The results are shown in figure 2. It is important to note that 75 % of crystals had an average size of $8.5 \pm 1.15 \mu\text{m}$, therefore less than $10 \mu\text{m}$, and 25% of the crystals had an average size of $5.56 \pm 0.87 \mu\text{m}$.

Example 2. Insulin protein in Di-alanine gel

Insulin protein in Di-alanine gel was prepared by sequentially dissolving the insulin to a final concentration of 5 mg/mL, in 6 mM HCl, 5 mM ZnCl₂, 22 mM Sodium citrate pH 7.0 and 0.2% di-alanine. The resulting preparation was subjected to 30 minutes of thermal shock at 4°C. The dose was then stored at 20°C. The di-alanine hydrogel was prepared by dissolving the peptide in DMSO, the DMSO being at a concentration of 15% of the final volume of the dose.

The results are shown in figure 2. It is important to note that 90.6 % of crystals had an average size of $10.28 \pm 2.13 \mu\text{m}$, therefore approximately $10 \mu\text{m}$, and 9.4 % of the crystals had an average size of $5.78 \pm 1.02 \mu\text{m}$.

Example 3. Comparative example

Hydrogels 1 (L) and 2 (D) (0.1% w/v) were prepared in MilliQ water by heating in a closed vial, as previously described. Hydrogels 3 (Di-Phe) and 4 (Di-Ala) (0.5% w/v) were prepared by dissolving the peptide in 5 μ L DMSO to a final concentration of 100 mg/mL followed by the addition of 100 μ L of MilliQ water. The excess of DMSO in the formed hydrogels was then removed by the addition of an excess of MilliQ water in each Eppendorf for 12 hours. This process was repeated several times for a week.

Insulin was dissolved in 6 mM HCl, 5 mM ZnCl₂ and 28 mM Sodium citrate pH 7.0.

Counter-diffusion technique with two layers configuration (2L) was used to set-up crystallization experiments in Eppendorf tubes. Two different set-ups were tested. In the first case the protein was allowed to diffuse within a pre-set hydrogel column (50 μ L) for one week while in the second case protein solutions were directly mixed with the hydrogel precursor to a final volume of 50 μ L, as explained above, and let it gel. Then, 50 μ L of the precipitant solution (30% acetone, 2 mM ZnCl₂, 28 mM sodium citrate, pH 7.0) were added on top of the hydrogel plus protein layer to start the crystallization experiment.

Experiments were stored at 20°C except for the insulin experiments that were incubated at 4°C.

The average size obtained by using this experiment is depicted in figure 1 (hydrogel 1(L) and 2(D)). The average size illustrated in figure 1 for agarose was obtained by using agarose as the gelator agent.

Example 4. Thermal stability and stability over time of hydrogel-grown insulin crystals

It is generally accepted that the maximum storage time for opened insulin vials is about 28 days at not more than 30°C, although this depends on the different commercial products and on the pharmaceutical companies producing them.

To check if the nature of the gel can affect insulin crystal stability, a series of experiments were designed in which the crystals grown in solution and in agarose and di-alanine hydrogels according to examples 1 and 2 above, were incubated at 4 temperatures (25°C, 40°C and 50°C) for 45 days. Preliminary observations (figure 5), based on a simple optical (microscopic) analysis of the appearance of the crystals and the emergence of particulate material, showed that insulin crystals grown in the hydrogels were stable, however, surprisingly di-alanine hydrogels were particularly stable at the different temperatures, withstanding such temperatures for 45 days up until now.

Example 5. Pharmacokinetic behavior of hydrogel-grown insulin crystals

The pharmacokinetic behaviour of the active ingredient in crystalline state is determined by the crystalline form of the active ingredient and by the gel in which the crystals were obtained.

5.1. Study of insulin bioactivity in supramolecular gels in vivo.

5 The purpose of the study was to evaluate the hypoglycemic effect of different human insulin preparations in supramolecular gels using Wistar rats as an experimental model. The use of this model is based on the relatively low inter-species specificity of insulin, such that human insulin is capable of stimulating insulin receptors in rats, just as porcine insulin is active in humans. For this purpose, two human insulin preparations in respective supramolecular agarose and di-

10 alanine gels prepared according to examples 1 and 2 above, as well as conventional human insulin in aqueous solution, were used. Given that the active ingredient is identical, the effect on the blood glucose profile fundamentally reflects pharmacokinetic differences, attributable in principle to the release of insulin in soluble form from the subcutaneous injection site.

5.1.2. Experimental model and protocol.

Male Wistar rats kept in standard conditions were used. The different forms of insulin were administered subcutaneously, with a maximum volume of 0.1 ml. Different blood samples were

20 taken over time by means of making a small cut at the tip of the rat's tail. 100 µl were placed in a pre-heparinized Eppendorf tube for subsequent glucose measurement. Blood glucose was determined by means of spectrophotometry using the SPINREACT GLUCOSE-LQ kit (St. Esteve de Bas, Gerona), using a 5-point calibration curve for higher precision.

25 The reference insulin was supplied by Sigma Aldrich (ref. 11376497001 ROCHE). In all cases, the insulin was prepared later in order to prevent the formation of possible precipitates.

Two different experimental protocols were used. An acute activity protocol (**PROTOCOL 1**, see fig. 6). was first applied, the details for which are provided below:

- 30
- Taking a baseline sample (immediately before administering anesthesia).
 - Administering anesthesia. Pentobarbital at a concentration of 50 mg/kg per rat was used intraperitoneally.
 - Subcutaneously administering the reference insulin reconstituted in sterile water.
 - 35 • Taking a sample every 20 minutes for the next 2 hours.
 - Centrifuging the blood at 360 g/5 min/4°C.

- Determining blood glucose
- Keeping the rest of the plasma at -80°C for possible insulin measurement by means of ELISA.

5 A dose-setting study was first conducted with the reference insulin using the preceding protocol. The objective was to establish a dose capable of producing a reproducible and robust lowering of blood glucose, but that at the same time does not produce substantial compensatory responses. It must be highlighted that the animals were not subjected to fasting during the first test. The experiments were always conducted in the morning at about the same time, around 10
10 am. Therefore, the baseline blood glucose of the rats must correspond to the normal level or slightly higher, given that they are a species that feeds throughout the night. The risk of excessively low blood glucose prior to setting the ideal dose is therefore reduced.

Two doses of 4.6 µg and 9.2 µg were administered, with n=3. The results of this experiment
15 were as follows t=0 corresponds to the baseline blood glucose before insulin administration, strictly speaking this would be a minimum time of 1-2 minutes. Significant compensatory responses above 60 mg/dl were not expected. Both insulin doses gave rise to a maximum drop in blood glucose that is comparable, without exceeding the level of 60 mg/dl, but the effect was more prolonged with the higher dose. Based on the foregoing, the dose of 9.2 µg was selected
20 for successive tests. (see fig.7)

The comparative test between the different forms of insulin was then carried out. To that end, several rounds were performed following the same protocol, and the data were grouped and then analyzed together. The experimental size was 8 for insulins in gel, and 6 for the reference
25 insulin. In order to reduce experimental noise, and once the insulin dose was established, the following experiments were conducted in 12-h fasting conditions.

The results obtained are illustrated in fig. 8. As expected, the reference insulin produced a hypoglycemic response comparable to that observed in the dose setting test, although it is more pronounced, presumably due to prior fasting. The maximum was reached between 20 and 40
30 minutes, according to the absorption profile known for this peptide. The agarose insulin had virtually identical profile, which probably reflects comparable absorption kinetics. In contrast, the di-alanine insulin did not show a measurable hypoglycemic effect. It was reasoning that the amounts of insulin released were very low to be detected. This implies that insulin in this form
35 has a slow release profile.

These experiments allow us to conclude that the agarose insulin had a pharmacological profile comparable in principle to that of the reference insulin, whereas the di-alanine insulin did not show significant activity at the dosages tested, pointing to a beneficial slow release profile.

5 To clarify if the di-alanine insulin was capable of producing pharmacological effects, additional experiments were conducted by modifying the protocol, (**PROTOCOL 2**, see fig. 9), to enable detecting, where appropriate, the hypoglycemic effect associated with ultraslow release of the active ingredient. To that end, higher doses and a prolonged follow-up time were used. The increase in dose tends to increase the amount of insulin reaching the blood stream, and
10 therefore the effect that can be observed on blood glucose. Even so, the effect to be expected within a short period of time (<1 h) is little; if there is a significant but sustained degree of release, a drop in blood glucose of moderate intensity, but for an extended time, would occur. Specifically, 32 h follow-up was performed. Given that it is not viable to keep animals without food or under anesthesia for such a long time, a protocol was carried out as described in detail
15 below:

- Removing food from the cages of the rats subjected to experiment.
- Taking a baseline sample.
- Subcutaneously administering insulin or carrier (di-alanine gel without insulin).
- 20 • Taking a sample 4 and 8 hours after insulin injection.
- Ending fasting after 8 hours: putting food in the cages.
- Removing food again 24 hours after administering the insulin.
- Taking a sample 28 and 32 hours after administration.

25 The protocol is described for greater clarity in fig. 9.

Doses that were 2x, 3x, 5x and 10x the initial crystallized insulin dose in Di-alanine gel were used. A significant drop in blood glucose (results not shown) was not observed with the administration of doses 2x, 3x, and 5x the initial dose. However, when using a 10x (i.e., 80 µg)
30 dose of insulin in Di-alanine hydrogel a lower blood glucose level was observed at 8 h, shown in fig. 10. As can be seen, the rats from both groups show an initial drop in blood glucose (after 4 hours) compared to the baseline value, attributable in principle to the removal of food, as well as a possible initial hyperglycemic effect associated with stress induced in the animals due to being handled. After 8 hours, a considerable recovery of blood glucose levels is seen in animals
35 from the control group, the reason for which is unknown, since the animals are still fasting at that point, but it is probably due to compensatory actions. In any case, blood glucose is lower in animals treated with di-alanine insulin, suggesting a sustained hypoglycemic effect. The effect was not detected at later time points, suggesting the effect is lost some time after 8 h.

To gain information pertaining the period of hypoglycemic effect suggested by the previous data, i.e. 8 h a new protocol (**PROTOCOL 3**, see fig. 11) was developed. Rats were fasted overnight (12 h) prior the administration of insulin. Firstly, blood samples were taking to
5 establish basal glycemic levels, followed by insulin administration. Blood samples were collected at 30 min, 1, 2, 4, 6 and 8 hours after injection. As expected, the data suggest that insulin dialanine effect may extend longer hours than reference insulin at the doses used (see fig. 12). Although these are preliminary results, they showed a slow release profile of the dialanine insulin from the site of injection. The release profile was quite similar than insulin
10 gargline (Lantus®) a well known basal insulin.

Example 6. Pharmacokinetic behavior of hydrogel-grown insulin crystals kept at 50 °C

Following the protocol 3 we tested the stability of reference insulin and insulin dialanine (5x and 10x doses) kept at 50 °C for a week. Three other groups of animals were included to complete
15 the study: Only dialanine gel; reference insulin; insulin dialanine.

The results (see fig. 13 for 5x dose and 14 for 10x dose) obtained indicate that, as expected, insulin dialanine had a longer hipoglycemic effect than the reference insulin. Reference insulin kept at 50 °C did not show any hipoglycemic effect while insulin dialanine kept at 50 °C showed
20 a similar hipoglycemic profile than the insulin dialanine although less pronounced.

These results show not only that the dialanine gel stabilize insulin at room temperature (see fig. 5) but also at 50 °C temperature with the retention of the 75% of activity aprox. An increase of protein stability implies an increase in the half-life and therefore, the improvement of the
25 bioavailability.

CLAIMS

1. A composition comprising a hydrogel which in turn comprises pharmaceutically active protein crystals grown *in-situ* within the hydrogel,

- wherein the hydrogel is a viscoelastic solid-like material comprised of an elastic cross-linked network and water, wherein water is the major component;

- wherein the pharmaceutically active protein crystals are composite materials of the protein in crystalline state having an average size of less than 12 μm measured by scanning electronic microscopy; and

- wherein the hydrogel can be either macromolecular or supramolecular; and

- wherein acetone is absent from the composition.

2. The composition of claim 1, wherein the hydrogel is macromolecular and comprises or consists of a compound selected from the group consisting of agarose, gelatin, carrageenan, Poly(ethylene glycol) (PEG).

3. The composition of claim 1, wherein the hydrogel is a supramolecular peptide-based hydrogel.

4. The composition of claim 3, wherein the supramolecular peptide-based hydrogel is a cysteine-based peptide or consists of peptides comprising an amino-acid chain of four or less amino-acids, preferably these peptides are di-peptides.

5. The composition of claim 4, wherein the di-peptide compound is a compound selected from the group consisting of Fmoc-CF-OH, Fmoc-MF-OH, Fmoc-FF-OH and Fmoc-AA-OH.

6. The composition of claim 2, wherein the macromolecular hydrogel is an agarose gel which comprises or consists of polysaccharides of agarobiose units, wherein agarobiose is a disaccharide formed by the union of D-galactose and 3,6-anhydro-L-galactose.

7. The composition of claim 5, wherein:

- the Fmoc-CF-OH hydrogel is based on the dipeptide N-(9-Fluorenylmethoxycarbonyl)-L-cysteine-L-phenylalanine;

- the Fmoc-MF-OH hydrogel is based on the dipeptide N-(9-Fluorenylmethoxycarbonyl)-L-methionine-L-phenylalanine;
- the Fmoc-FF-OH hydrogel is based on the dipeptide N-(9-Fluorenylmethoxycarbonyl)-L-phenylalanine -L-phenylalanine; and
- 5 - the Fmoc-AA-OH hydrogel is based on the dipeptide N-(9-Fluorenylmethoxycarbonyl)-L-alanine -L-alanine.

8. The composition of claim 2, wherein the macromolecular hydrogel is PEG hydrogel which is composed of poly(ethylene glycol) monomethyl ether monomethacrylate (PEGMA) of average
10 molecular weight of 1100 Da cross-linked with poly(ethylene glycol) dimethacrylate (PEGDMA) of MW 1200 Da.

9. The composition of claim 4, wherein the cysteine-based peptide is N, N'-di(benzoyl)-cysteine-diamide.

15 10. The composition of any of the precedent claims, wherein the pharmaceutically active protein crystals are selected from the list consisting of any of the following compounds: insulin, lysozyme, albumins, phosphatase alkaline, glucose isomerase, growth hormone, somatotropin, factor VIII, factor IX, antithrombin III, immunoglobulins, erythropoietin, interferons, papain,
20 trypsin, hyaluronan-degrading enzyme, collagenase, streptokinase, glucagon, thyrotropin, secretin, humanized chimeric mAB (monoclonal antibodies), vaccines, HIV antigens and hepatitis C antigens.

25 11. The composition of claim 10, wherein the pharmaceutically active protein crystals are insulin crystals.

12. The composition of claim 11, wherein the hydrogel is a peptide-based hydrogel formed by Fmoc-AA-OH or an agarose gel comprising polysaccharides of agarobiose units.

30 13. The composition of claim 11, wherein the hydrogel is formed by PEG with an average MW 1100 Da.

14. A process to manufacture a composition comprising a hydrogel comprising pharmaceutically active protein crystals grown *in-situ* within the hydrogel, wherein the pharmaceutically active
35 protein crystals grown *in-situ* have an average size of less than 12µm; which comprises:

- a. Sequentially blending a pharmaceutically active protein solution in their appropriate buffer composition by using a value of supersaturation in the metastable zone width

(MSZW) or nearby the supersolubility curve, with a precipitant solution, wherein acetone is absent, and a gelator solution,

b. Inducing the nucleation by lowering the temperature, and

c. Storing the resulting mix at a constant temperature, preferably at room temperature at 1 atm.

15. The process of claim 14, wherein the protein active solution is a solution comprising insulin, wherein the precipitant solution is HCl, ZnCl₂ and sodium citrate, wherein the gelator solution is the Fmoc-AA-OH hydrogel and wherein the induction of the nucleation is performed by placing the product resultant from step a) at approximately 4°C, preferably for about 30 minutes.

16. The process of claim 14, wherein the protein active solution is an insulin solution, wherein the precipitant solution is HCl, ZnCl₂, DMSO and sodium citrate, wherein the gelator solution is agarose and wherein the induction of the nucleation is carried out by allowing the product resultant from step a) to reach room temperature or actively cooling it to room temperature.

17. The composition of any of claims 1 to 13, wherein said composition is a pharmaceutical composition.

18. The pharmaceutical composition of claim 17, for use in the controlled liberation of pharmaceutically active protein crystals.

19. The composition of any of claims 11 to 13, wherein said composition is a pharmaceutical composition.

20. The pharmaceutical composition of claim 17, for use in the controlled liberation of pharmaceutically active protein insulin crystals.

21. The pharmaceutical composition of claim 17 or 19, for use in therapy.

22. The pharmaceutical composition of claim 19, for use in the therapeutic treatment of diabetes.

FIGURES

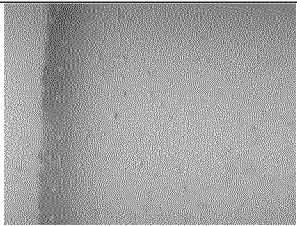
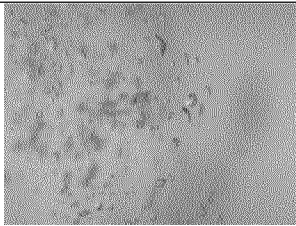
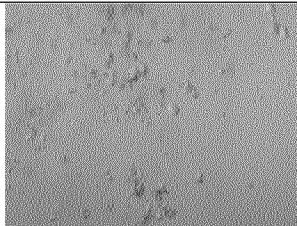
Agarose gel	D Gel	L Gel
		
Maximum length measured	Maximum length measured	Maximum length measured
36.1 x 23 microns	96.5 x 53.9 microns	40,2 x 35.8 microns
Minimum length measured	Minimum length measured	Minimum length measured
16.4 x 12.9 microns	21.6 x 19.2 microns	21.6 x 10.8 microns

Fig. 1

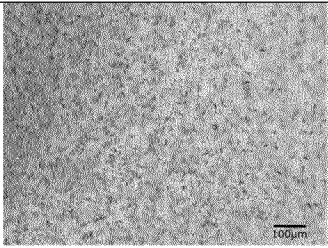
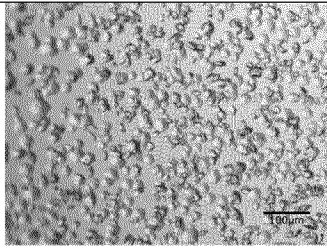
Agarose gel + DMSO	Di-AlaDiAla gel
	
25 % of crystals had an average size of $5.56 \pm 0.87 \mu\text{m}$	9.4 % of crystals had an average size of $5.78 \pm 1.02 \mu\text{m}$
75 % of crystals had an average size of $8.5 \pm 1.15 \mu\text{m}$	90.6 % had an average size of $10.28 \pm 2.13 \mu\text{m}$

Fig. 2

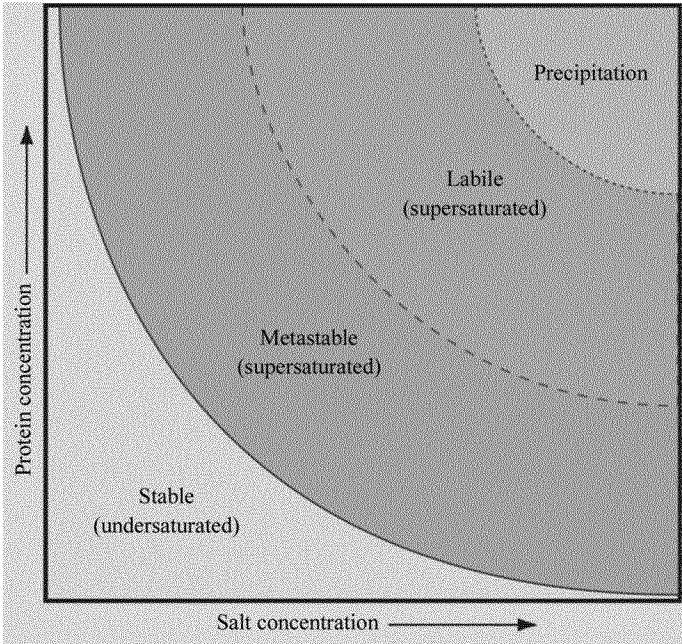


Fig. 3

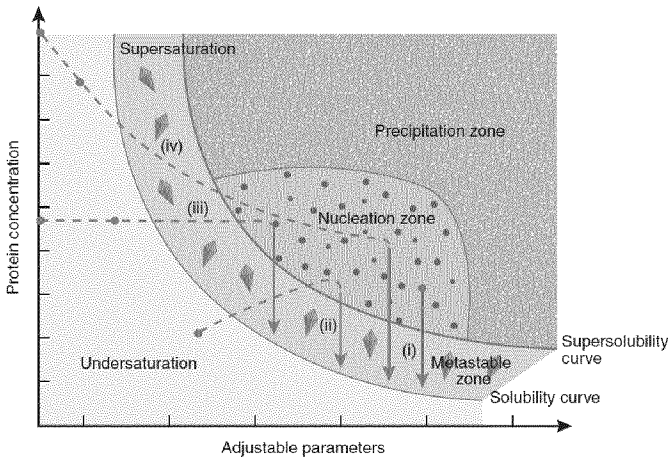


Fig. 4

Temperature	Solution	Crystals in solution	Crystals in agarose	Crystals in Di-alanine
Initially prepared dose			Microcrystals	Microcrystals
25 degrees	Clear drop	Faceless crystals	Dissolved microcrystals	Some crystals with perfect faces
40 degrees	Clear drop	Dissolved crystals and precipitate	Precipitation/Aggregation and denaturation	Some crystals with perfect faces
50 degrees	Clear drop	Dense precipitate and denaturation	Precipitation/Aggregation and denaturation	Some crystals with perfect faces

Fig. 5

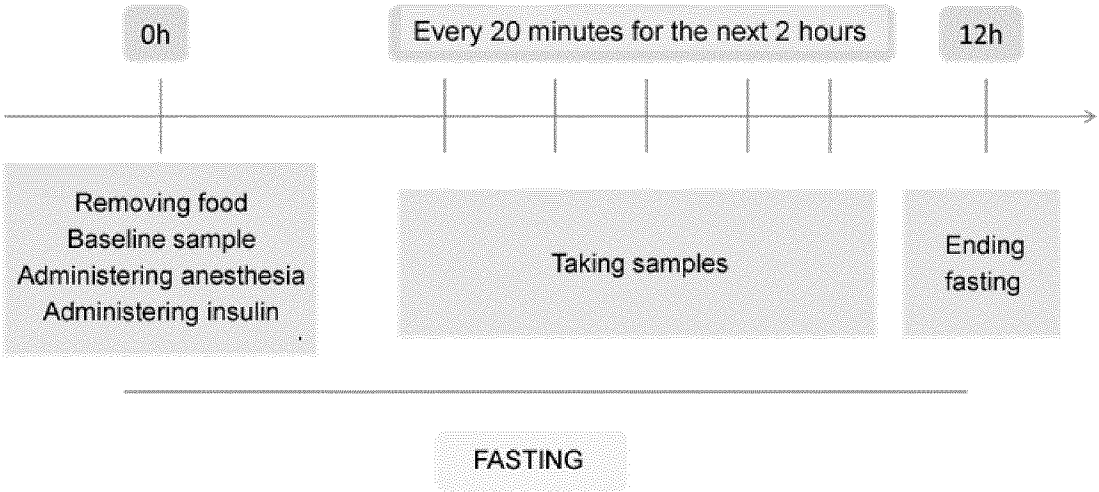


Fig. 6

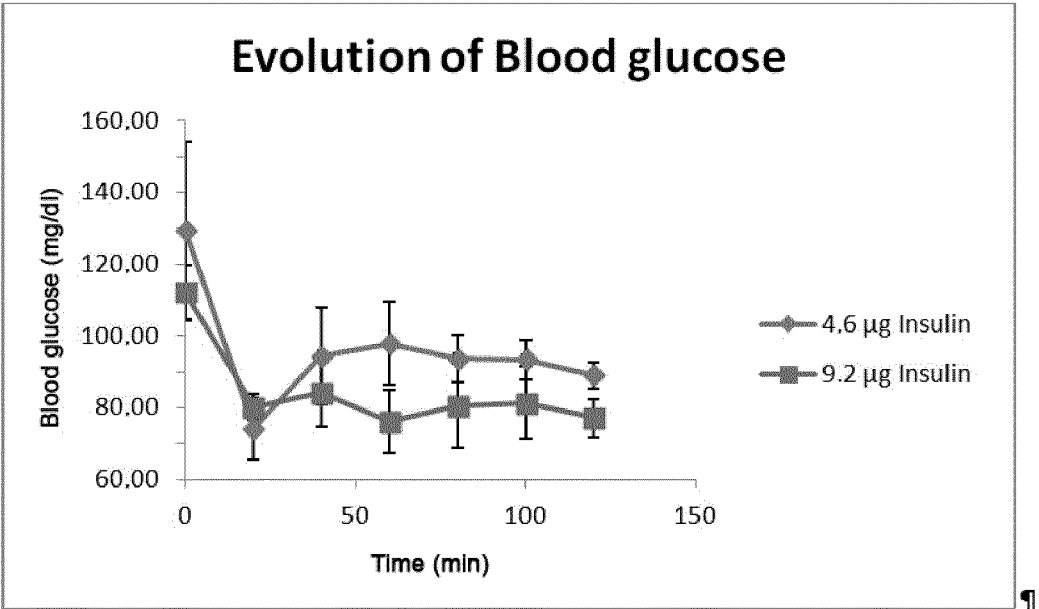


Fig 7.

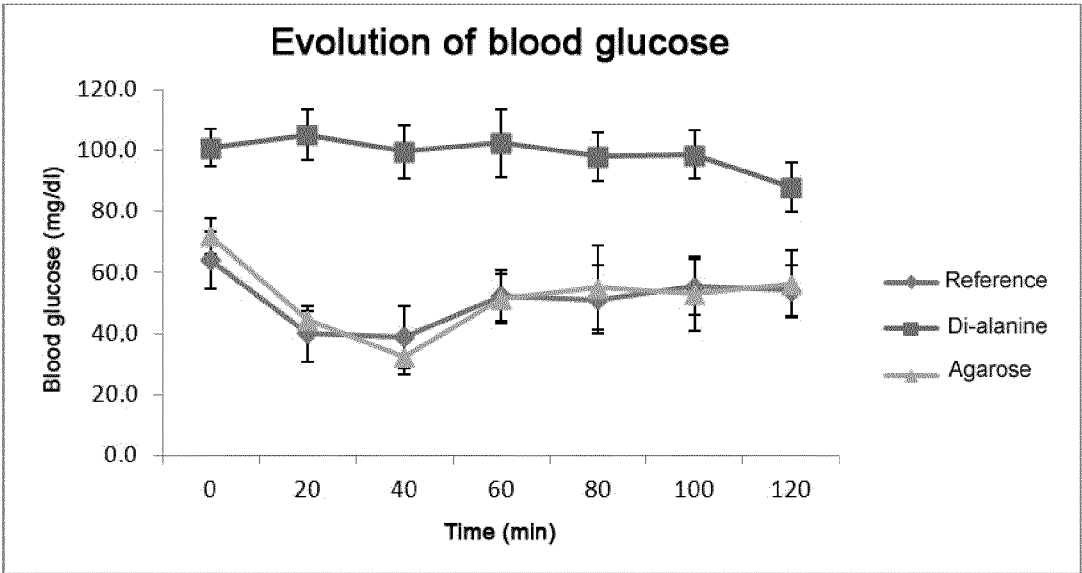


Fig 8.

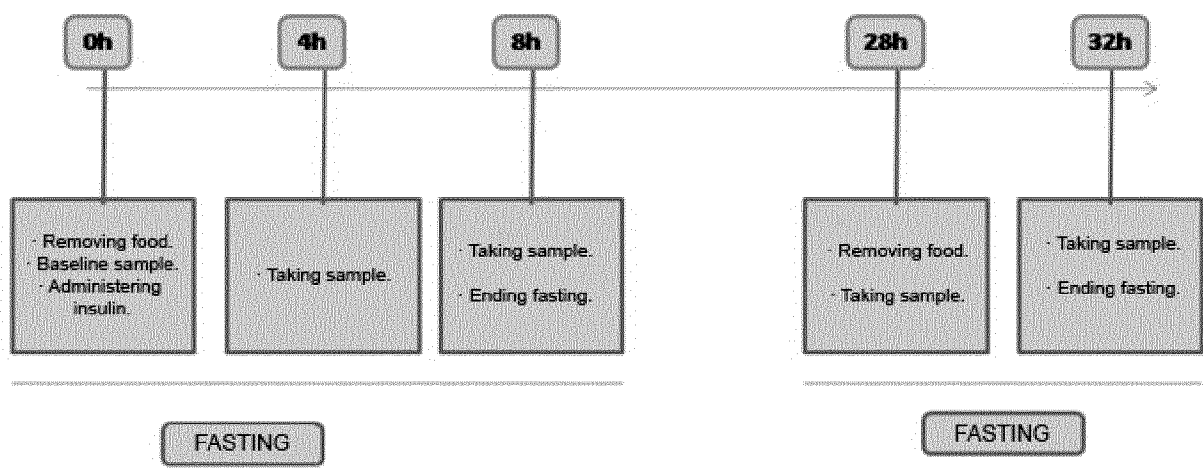


Fig. 9

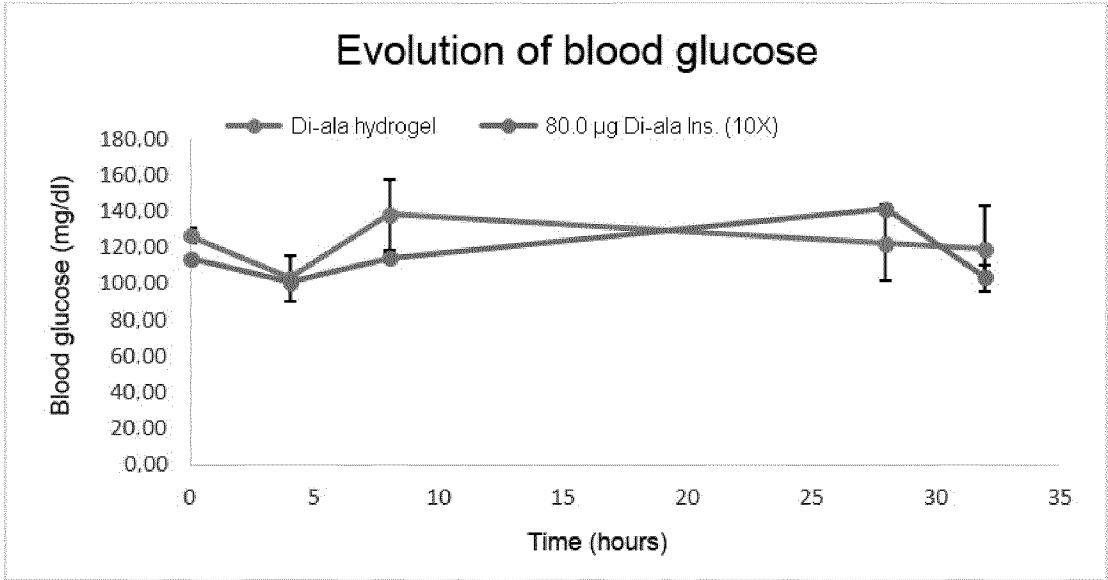


Fig. 10

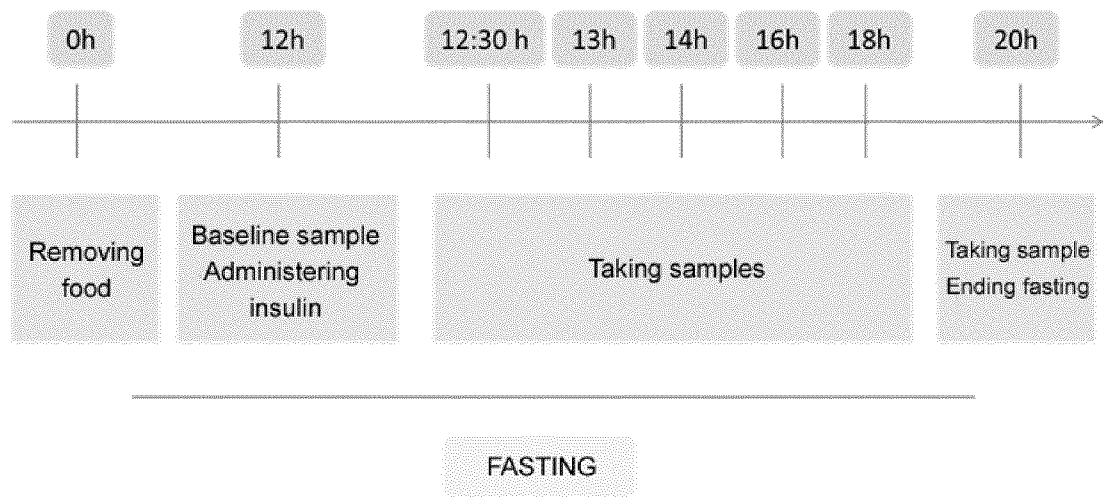


Fig. 11

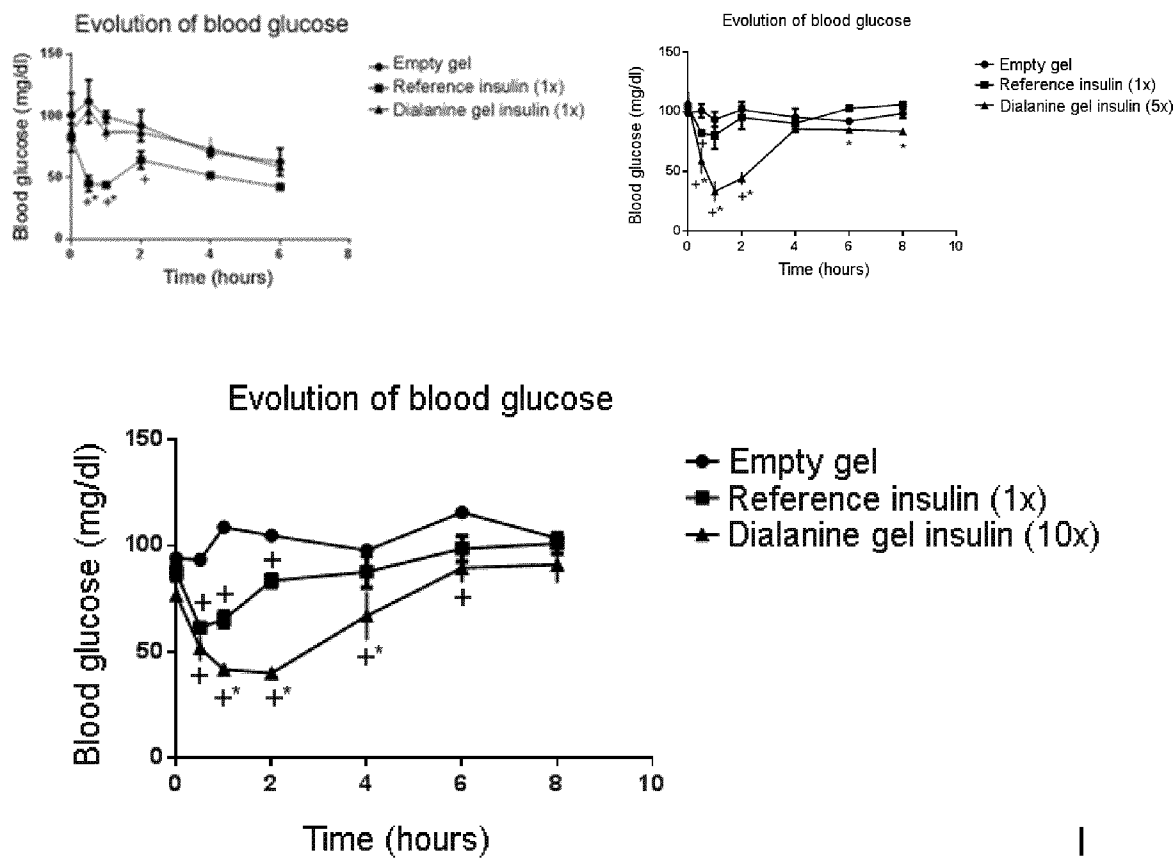


Fig. 12

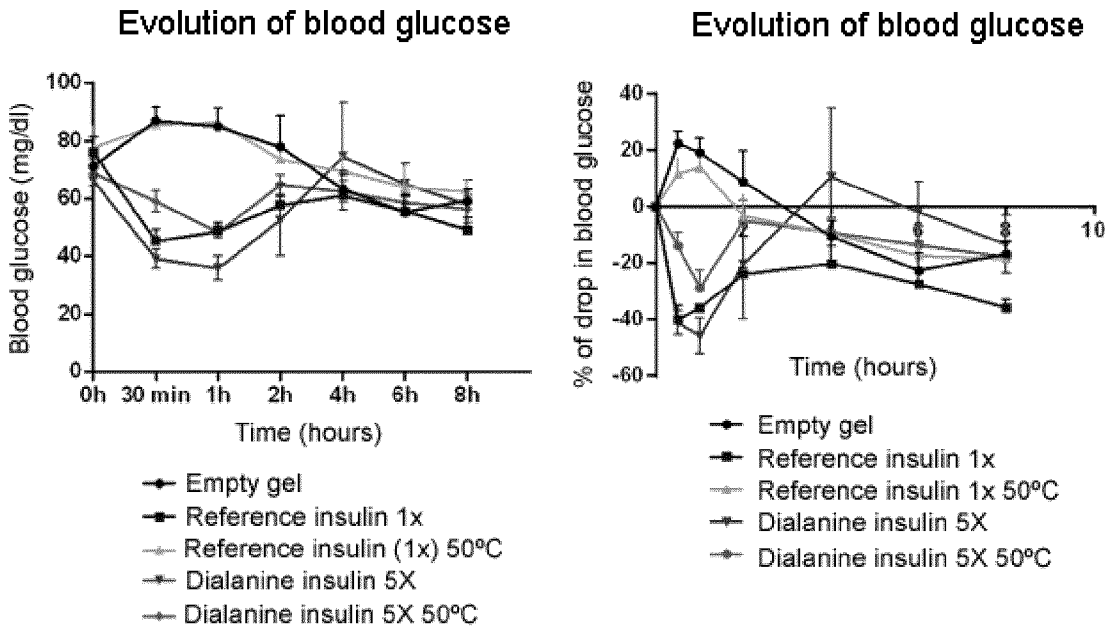


Fig.13:

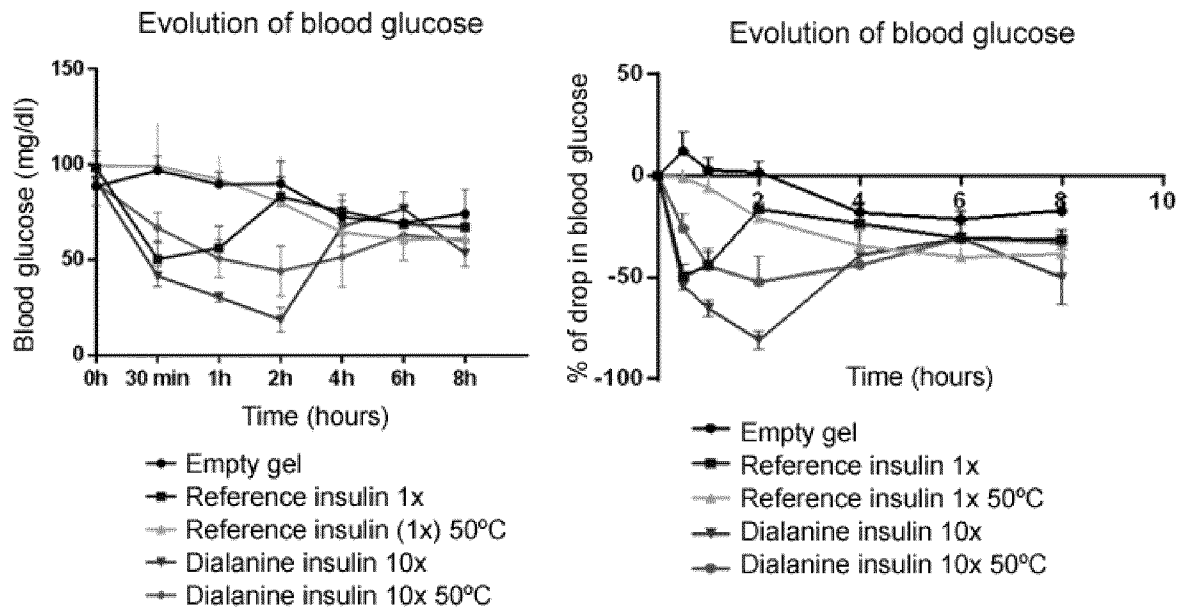


Fig.14

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/060842

A. CLASSIFICATION OF SUBJECT MATTER INV. A61L27/52 A61K38/28 A61K38/52 A61K47/18 A61K47/42 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) A61L 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, WPI Data, BIOSIS, EMBASE		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MAYTE CONEJERO-MURIEL ET AL: "Protein crystallization in short-peptide supramolecular hydrogels: a versatile strategy towards biotechnological composite materials", CRYSTENGCOMM, vol. 17, no. 42, 1 January 2015 (2015-01-01), pages 8072-8078, XP055383480, GB ISSN: 1466-8033, DOI: 10.1039/C5CE00850F the whole document figure 6 -/-	1-22
☒ Further documents are listed in the continuation of Box C. ☒ 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 "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "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 "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 22 June 2017		Date of mailing of the international search report 05/07/2017
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Schnack, Anne

INTERNATIONAL SEARCH REPORT

International application No

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X	SHIGERU SUGIYAMA ET AL: "Development of protein seed crystals reinforced with high-strength hydrogels", CRYSTENGCOMM, vol. 17, no. 42, 1 January 2015 (2015-01-01), pages 8064-8071, XP055383503, GB ISSN: 1466-8033, DOI: 10.1039/C5CE00844A page 8065 -----	1-22
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

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