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(72) Inventors; and

(71) Applicants: GÓMEZ, Marité Cárdenas [SE/ES]; Calle Peña de Santa Marina 31G, 48993 Getxo (ES). DEL GIUDICE, Rita [IT/SE]; Barlastgatan 4, 216 49 Limhamn (SE). KANAFUSA, Sumiyo [JP/SE]; Basungränden 14D, 22468 Lund (SE).

(72) Inventor: LARSSON, Johan; Christians Gränd 8, 226 39 Lund (SE).

(74) Agent: HØIBERG P/S; Adelgade 12, 1304 Copenhagen K (DK).

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(54) Title: NANOCAPSULES FOR ENCAPSULATION AND DELIVERX OF WATER SOLUBLE COMPOUNDS

(57) Abstract: The present invention relates to a dispersion for encapsulation and delivery of added value compounds. The dispersions comprise: casein, wherein at least 60% of the casein is κ -casein; and phospholipids.

NANOCAPSULES FOR ENCAPSULATION AND DELIVERX OF WATER SOLUBLE COMPOUNDS

Technical field

The present invention relates to dispersions such as nanocapsules and emulsions useful for as carrier for added value compounds, for stabilizing
5 added value compounds and/or for stabilizing food products and/or beverages. The invention also relates to nanocapsules for encapsulation and delivery of added value compounds, such as water-soluble nutrients to cheese and to a method for producing the dispersions or the nanocapsules. The present invention further relates to a method for producing food products, such as a
10 fortified cheese and/or a cheese with added functionality or beverages, such as fortified juice or milk.

Background

Nanotechnology has increasingly been employed in food science,
15 specifically in the development of functional food products where enhanced delivery of nutrients and bioactive compounds is desirable. Among the various carriers used, nanocapsules and emulsions have garnered attention for their ability to encapsulate, protect, and deliver both hydrophilic and hydrophobic compounds. Emulsions further offer the advantage improving the sensory
20 qualities of food.

Despite the advancements in nanotechnology applications in food, challenges remain in efficiently encapsulating both hydrophilic and hydrophobic substances within a single system while maintaining stability under different environmental conditions. Additionally, the use of natural and food-grade
25 materials for encapsulation is crucial for consumer safety and acceptance.

Cheese is a dairy product that comprises proteins and fat from milk. The process for cheese making consists of several steps. Briefly, pasteurized milk which contains casein micelles is slowly heated, acidified, and inoculated with a starter culture in the presence of a renneting enzyme under gentle stirring.
30 The casein micelles consist of alpha- and beta-caseins enclosed and stabilized by kappa(κ)-casein. During this so-called coagulation phase, the hydrophilic

and hydrophobic ends of the κ -caseins are cleaved by the renneting enzyme leading to coagulation and formation of cheese curd. After a short incubation time, the cheese curd is cut into small pieces and syneresis (dehydration phase) takes place while the mix containing solid curd and liquid whey is
5 swirled. The whey, which is a supernatant rich in water soluble compounds, is then removed from the curd and the curd is pressed overnight at room temperature to obtain the final cheese mass. During the so-called ripening phase, that goes from two weeks up to two years, the mature cheese is finally formed thanks to slow enzymatic activity of proteases and lipases.

10 Cheese is valued for its portability, long shelf life, and high content of fat, protein, calcium, and phosphorus. Cheese is furthermore one of the best diet sources of calcium, and vitamin B12 is typically abundant in cheese. However, for the sake of the aging population and for the fact that today people rely on a reduced consumption of animal products, the nutritional value of cheese could
15 be further improved to include antioxidants, vitamins, and minerals, such as iron.

Cheese could also be produced containing enzymes, such as the ones needed to metabolize lactose (beta-galactosidase), and to promote digestion of fat and complex carbohydrates (lipases and amylases). Such compounds
20 are either themselves essential or enable adsorption of essential compounds for human metabolism. Furthermore, their addition to food products is particularly important for those found in insufficient amounts in a normal diet (especially important for vegetarians). Beta-galactosidase supplementation would benefit lactose intolerants whereas lipases and amylases
25 supplementation favour food digestion in general but is particularly beneficial for individuals with exocrine pancreatic insufficiency. Such enrichment with enzymes will give cheese an added functionality.

Still, there are some major limitations for achieving this purpose in cheese making. First, cheese is an animal milk product and, as such, its final
30 nutritional properties mirror those of the type of milk used. Additionally, an important technological consequence of cheese making is that

supplementation with water soluble compounds is limited because these components are lost in the whey during the process of cheese making.

High-value nutrients supplementation is even more important when considering the high market interest of vegan cheese. Vegan cheese currently
5 on the market, apart from lacking the organoleptic properties, e.g. taste and texture, of dairy cheese, also lacks many nutritional elements. Fortification of vegan cheese is thus needed to boost its nutritional levels for the sake of the vegan population.

Previously, casein micelles have been used to encapsulate iron and to
10 fortify cheese (Raouche et al. 2009). However, the iron used in this case is derived from iron-containing salts, which are not as easily up-taken by the body and metabolized. For this reason, the amount of iron used was high and the cheese obtained had a red-brownish color and unpleasant taste. Moreover, the presence of iron contributed to the oxidation and instability of milk.

15 US 2006/0246180 discloses a process for natural cheese-making. Cheese fortification during this process may occur at three locations: the first fortification being after separation of the curds and whey, the second fortification being supplementing the brine solution with fortificant, and a final fortification being prior to or during the final packaging. A drawback of this
20 process is that the fortificants are applied after rennet addition and curd formation. This results in that the fortificants are poorly incorporated or protected by the curd structure.

Therefore, there is a need for dispersions prepared from food grade materials useful as carrier for added value compounds, for stabilizing added
25 value compounds and/or for stabilizing emulsions. In addition, there is a need for a reliable and improved method for fortification of cheese, which method results in a fortified cheese, with good structure and taste, and can be used to add new health promoting functionality to the cheese.

30 **Summary of the invention**

Considering the above, the present invention aims to solve the problems of the prior art. This invention aims to utilize the unique properties of κ -casein

and specific phospholipids to develop novel dispersions, such as nanocapsules and emulsions. These systems are designed to enhance the encapsulation efficiency, stability, and delivery of added value compounds in food products, and provide effective delivery systems suitable for a wide range of applications
5 in the food and pharmaceutical industries.

The inventors have identified κ -casein, a subtype of casein, as especially effective in forming stable dispersions with phospholipids, due to its unique molecular properties. Thus, the present invention provides dispersions made from κ -casein and phospholipids. The dispersions can be useful for
10 multiple purposes including as carrier for added value compounds and/or for stabilizing emulsions. The dispersions of the invention can be particularly useful for delivering added value compounds in vivo, because of their stability under conditions mimicking the gastric environment, but they may also have high utility in manufacture of cheese or cheese analogues. In addition, the
15 dispersions can be useful for stabilizing added value compounds in certain environments, for example in milk and other beverages, such as fruit juice.

Dispersions comprising a high percentage of κ -casein loose colloidal stability in the presence of calcium chloride and precipitate in the presence of chymosin, which render such dispersions particularly useful for production of
20 cheese or cheese analogues, because the dispersion can carry added value compounds, and release them into the cheese during production.

Thus, a first aspect of the invention relates to a dispersion, said dispersion comprising:

- 25 a) casein, wherein at least 60% of the casein is κ -casein; and
 b) phospholipids.

Dependent on various parameters, for example the production method, the dispersions may take different forms. For example, the dispersion may be in
30 the form of nanocapsules or emulsions. Nanocapsules typically comprise a shell of phospholipid and κ -casein encapsulating a hydrophilic core. Nanocapsules are typically prepared by preparing a lipid film, preparing an

aqueous solution comprising κ -casein and optionally hydrophilic added value compounds, and solubilizing the lipid film in the solution. Nanocapsules of the invention are particularly useful as carriers for hydrophilic added value compounds. Emulsions typically comprise oil in water droplets comprising a
5 shell of κ -casein and a hydrophobic core. Optionally, the emulsions comprise further phospholipids. Emulsions may be prepared from a hydrophobic phase optionally comprising an added value compound, and mixing with an aqueous solution comprising κ -casein. Emulsions of the invention are particularly useful as stabilizers for food products and/or beverages and/or as carriers
10 for hydrophobic added value compounds.

Accordingly, a second aspect of the invention relates to a nanocapsule comprising:

- 15 a) a shell comprising phospholipid and casein, wherein at least 60% of the casein is κ -casein; and
- b) a hydrophilic core.

A third aspect of the invention relates to an emulsion comprising oil in water droplets comprising:

- 20 a) a shell comprising casein, wherein at least 60% of the casein is κ -casein and optionally phospholipid; and
- b) a hydrophobic core.

A fourth aspect of the invention relates to method of producing the
25 nanocapsules as described herein, wherein the method comprises the following steps:

- a) preparing a lipid film;
- b) preparing a hydrophilic solution comprising casein, wherein at
30 least 60% of the casein is κ -casein and optionally an added value compound;
- c) solubilizing said lipid film prepared in step a) in the solution prepared in step b); and
- d) obtaining a solution containing the nanocapsules.

A fifth aspect of the invention relates to a method of producing the emulsion as described herein, wherein the method comprises the following steps:

- 5 a) providing a hydrophobic solution, preferably comprising an oil and/or phospholipid;
- b) preparing a hydrophilic solution comprising casein, wherein at least 60% of the casein is κ -casein;
- c) Emulsifying said hydrophobic solution with said casein solution;
- 10 d) thereby obtaining an emulsion.

A sixth aspect of the invention relates to a use of the dispersion, the nanocapsules or the emulsion as described herein for the transport and/or encapsulation of added value compound(s).

- 15 A seventh aspect of the invention relates to a use of the dispersion, the nanocapsules or the emulsion as described herein for enrichment of a food product and/or beverages with an added value compound.

- 20 An eighth aspect of the invention relates to of the dispersion, the nanocapsules or the emulsion as described herein for increasing the nutritional value in food products, such as cheese, cheese analogues and/or beverages.

A ninth aspect of the invention relates to method of producing a food product and/or beverages, wherein the method comprises the following steps:

- 25 a) Providing a food product, a beverage, food product ingredient or a beverage base;
- b) contacting said food product, beverage, food product ingredient or beverage base with the dispersion, the nanocapsules or the emulsion as described herein,
- 30 c) processing the mixture of step b) into a product or a beverage.

A tenth aspect of the invention relates to method of producing cheese or cheese analogue, wherein the method comprises the following steps:

- a) Providing milk or milk analogue comprising the dispersion, the nanocapsules or the emulsion as described herein;
- b) processing said milk or milk analogue into cheese or cheese analogue.

5

Description of Drawings

Figure 1: illustrates a scheme for generation of useful sources of κ -casein that for example can be used to make nanocapsules and emulsions.

10

Figure 2: shows a multiple sequence alignment of κ -casein from different organisms. The alignment has been generated by using CLUSTAL W (1.83).

Figure 3: illustrate that capsules can be made using κ -casein produced in *E. coli* A) SDS-PAGE of recombinant donkey κ -casein purified from *E. coli* protein extract by cationic exchange chromatography B) On the left - Nanocapsules made with recombinant donkey κ -casein produced in *E. coli* (0.5 mg/mL Lactoferrin, 0.8 mg/mL κ -casein). On the right - Emulsion made with recombinant donkey κ -casein produced in *E. coli* (4 mg/mL κ -casein, 3.5 vol% oil and 15 mg/mL lecithin).

20

Figure 4: illustrates that emulsions can be made using κ -casein produced in *C. reinhardtii* A) Western Blot showing the presence of bovine κ -casein in protein extract from *C. reinhardtii* B) Emulsions made with recombinant κ casein produced in microalgae (70 mg/mL algae protein including κ -casein)

25

Figure 5: shows an example of a useful scheme for production of κ -casein stabilized nanocapsules. The scheme shows a method to produce a nanocapsule for encapsulation and delivery of water-soluble added value compounds to cheese according to an embodiment of the inventive concept.

30

The figure shows a method to produce a nanocapsule for encapsulation and delivery of water-soluble nutrients to cheese. The method comprising the steps:

- a) Preparing a lipid film by dissolving phospholipids in an organic solvent, such as chloroform or hexane, and evaporating the organic solvent.
- 5 The phospholipid may be synthetic phospholipid, animal-based phospholipid and/or plant-based phospholipid. One example of phospholipids is lecithin. The lecithin may be both animal lecithin and plant based lecithin. Non-limiting examples of animal lecithin are egg yolk lecithin and milk lecithin. Non-limiting examples of plant based lecithin are soybean lecithin, corn lecithin, rapeseed
- 10 lecithin, sunflower lecithin, cotton seed lecithin and grain lecithin. The lecithin may be pure lecithin or food grade lecithin.

The evaporation may be conducted under vacuum using a desiccator or a plate evaporator. It should be noted that any type of solvent evaporator may be used for evaporation of the organic solvent.

- 15 b) Preparing an aqueous solution comprising at least one water-soluble added value compound.
- c) Preparing a κ -casein solution.
- d) Solubilizing the lipid film prepared in step a) by adding to and mixing with the solutions prepared in step b).
- 20 e) Adding the κ -casein solution obtained in step c) thus obtaining a solution containing nanocapsules comprising a shell made of phospholipid and κ -casein and a core comprising at least one water-soluble added value compound. Step e) may occur simultaneously with or after step d)

In case the water-soluble compound is a protein, the protein

25 concentration of step b) may be up to 10 mg/mL, preferably from 3 to 6 mg/mL. The concentration of κ -casein in the κ -casein solution of step c) may be up to 20 mg/mL, preferably from 1 to 10 mg/mL.

In case the water-soluble compound is a vitamin, the vitamin

concentration of step b) may be up to 20 mg/mL, preferably from 1 to 5 mg/mL.

30 The concentration of phospholipids in the solubilized lipid film of step d) may be from 0.1 up to 20 mg/mL, preferably 10 mg/mL.

The method may further comprise the steps of:

a') providing a protein powder comprising κ -casein and at least one water-soluble added value compound, and

b') suspending the protein powder in a buffer solution containing imidazole or Bis-Tris and sodium chloride, wherein steps a' and b' occur before
5 step b). The concentration of imidazole or Bis-Tris in the buffer solution may be between 20 and 25 mM. The concentration of sodium chloride in the buffer solution may be between 20 and 150 mM.

In step d), a solution comprising nanocapsules and excess of κ -caseins and/or phospholipids is obtained. In order to provide nanocapsules of good
10 quality, it is important that mixing is good. To this end, step d) may comprise mixing via vortexing, sonication or homogenization for solubilization of the lipid film.

Figure 6: shows an example of a useful scheme for production of κ -casein
15 stabilized emulsions. The scheme shows a method to produce an emulsion, which for example can be used for making milk or milk analogues and/or can be used for encapsulation and/or delivery of hydrophobic added value compounds to food products and/or beverages. The method may comprise the steps:

20 a) Preparing a hydrophobic solution, for example comprising an oil comprising phospholipid and optionally at least one hydrophobic added value compound. The oil may be olive oil, rapeseed oil, soybean oil, sunflower oil or corn oil. The phospholipid may be synthetic phospholipid, animal-based phospholipid and/or plant-based phospholipid. One example of phospholipids
25 is lecithin. The lecithin may be both animal lecithin plant-based lecithin. Non-limiting examples of animal lecithin are egg yolk lecithin and milk lecithin. Non-limiting examples of plant-based lecithin are soybean lecithin, corn lecithin, rapeseed lecithin, sunflower lecithin, cotton seed lecithin and grain lecithin. The lecithin may be pure lecithin or food grade lecithin.

30 b) Preparing an aqueous solution comprising κ -casein.

c) Preparing emulsion with the hydrophobic solution prepared in step a) by homogenizing and mixing with the aqueous phase solutions prepared in step b).

Thus, a solution may be obtained containing emulsions comprising a shell made of phospholipid and k-casein and a core optionally comprising at least one hydrophobic added value compound.

In case the hydrophobic compound is a vitamin or an antioxidant, the
5 concentration of step a) may be up to 20 mg/mL, preferably from 0.1 to 10 mg/mL. The concentration of oil of step a) may be up to 20 vol %, preferably 0.5- 10 vol %. The concentration of phospholipids of step a) may be up to 20 mg/mL, preferably 10- 15 mg/mL.

The concentration of k-casein in the k-casein solution of step b) may be
10 up to 40 mg/mL, preferably from 20 to 40 mg/mL.

The method may further comprise the steps of:

a') providing a protein powder comprising k-casein, containing imidazole or BisTris and sodium chloride. The concentration of imidazole or Bis-Tris in the buffer solution may be between 10 and 40 mM, preferably 20-25 mM. The
15 concentration of sodium chloride in the buffer solution may be between 20 and 150 mM, preferably 20-60 mM.

In step c), a solution comprising emulsions and excess of k-casein and/or phospholipids is obtained. Step c) may comprise mixing via vortexing, sonication or homogenization.
20

Figure 7: shows examples of useful schemes for methods for various food fortification. The upper part illustrates a method for production of fortified cheese according to an embodiment of the inventive concept.

The figure illustrates a method for production of fortified cheese and/or cheese
25 with added functionality. The method comprising the steps of:

- a) providing nanocapsules comprising a shell comprising phospholipid and k-casein, and a core comprising at least one water-soluble added value compound;
- b) adding the nanocapsules to milk thus forming fortified milk;
- 30 c) adding renneting enzymes to the fortified milk thus forming fortified cheese curd and whey;
- d) separating the fortified cheese curd from the whey; and
- e) pressing the fortified cheese curd into cheese.

In the present method, the nanocapsules are added with the milk and thereafter renneting enzymes are added to form the cheese curd. An effect of this is that upon renneting, the nanocapsules coagulate together with the caseins in the milk and form a cheese curd with incorporated and protected water-soluble
5 nutrients. The water-soluble added-value compound thus becomes incorporated and protected by the nanocapsules embedded in the cheese curd. The milk used in the method may comprise plant-based milk and/or dairy milk. The milk may comprise between 2.9 and 3.1% by weight of fat. The milk may be non-homogenized milk.

- 10 The renneting enzymes, such as chymosin, may be added in amount of typically, 0.2 U/mL to the fortified milk.

Optionally, the method may comprise a step of: f) enriching the milk with calcium chloride, wherein the step f) occurs after step b). The method may further comprise a step of: g) acidifying the milk by adding an acid, such as
15 citric acid, or by adding starter, such as lactic acid bacteria, to the milk, wherein the step g) occurs after step b).

After possible enriching and acidifying, equilibration may take place. The equilibration may be conducted at e.g. 32 °C for 20 minutes. Incubation may take place at 32 °C for 15 minutes.

- 20 Thereafter the curd is cut, and further incubation may take place at e.g. 32°C for 15 min.

During the incubation process, the curd may be cut in 5 minutes intervals. The cheese mass is then separated from whey, e.g. by filtration.

- 25 **Figure 8:** illustrates dynamic light scattering (DLS) measurements showing that nanocapsules are a single type of particle that differ from that of its single components: lecithin, casein or the cargo protein lactoferrin. Concentrations used are: 2 mg/mL lactoferrin, 2 mg/mL κ-casein and 10 mg/mL lecithin. The figure illustrates results from DLS showing that nanocapsules are highly
30 efficient in incorporating lactoferrin. Lactoferrin is used to fortify cheese with bioavailable iron. The curve representing nanocapsules made by the method

according to the inventive concept is larger than the curve representing free casein, lactoferrin and lecithin liposomes, respectively.

Figure 9: illustrates DLS data showing that nanocapsules can be made with
5 plant-based purified lecithin from soy or sunflower, and also with food graded
lecithin. In these experiments, 0.2 mg/mL lecithin was used in 25 mM Bis-Tris
buffer pH 6.7 enriched with 25 mM NaCl. Other phospholipid sources besides
natural lecithin of animal or plant origin can be used including purified
phospholipid type groups (e.g. phosphatidylcholine) or specific synthetic
10 phospholipids.

Figure 10: illustrates DLS measurements showing that nanocapsules are
stable at A) pH 4.6 to 6.7 and B) in different salinity between 0 to 100 mM NaCl
in imidazole buffer at pH 6.5. Concentrations used are: A) 0.5 mg/ml soy
15 lecithin, B) 1 mg/ml soy lecithin, A+B) 1.36 mg/mL κ -casein.

Figure 11: illustrates cryogenic transmission electron microscopy images and
small angle X ray scattering data and best fit for a nanocapsule according to
the inventive concept, showing the shell-core structure of the nanocapsule. The
20 figure shows structural confirmation of the nanocapsule wall being composed
of lipid bilayer and κ casein. The inner part of the capsule is made of water. A)
CryoEM and B) Small Angle Xray Scattering (SAXS) data and best fit using
core multi shell model: aqueous core, lipid head, lipid tails, lipid head/ κ -casein.
The results from Cryo-TEM and SAXS show that the nanocapsules have a
25 core-shell structure, in which the core is water filled and the shell is composed
of phospholipids and κ -casein.

Figure 12: illustrates stability of the nanocapsules upon storage at 4 °C (left
panels) and 25 °C (right panels) as measured by DLS (top) and visual
30 inspection (bottom) of the samples. The nanocapsules are stable for at least
up to 38 weeks at 4 °C and 5 weeks at 25 °C. Concentrations used are: 2

mg/mL lactoferrin, 2 mg/mL κ -casein in 20 mM imidazole, 20 mM NaCl buffer pH 6.5.

- Figure 13:** illustrates stability of the nanocapsules upon freeze drying and after resuspension in water as measured by DLS (top) and visual inspection (bottom) of the samples. The samples are stable at least after 7 weeks of re-suspension and storage at 4 °C. Concentrations used are: 2 mg/mL lactoferrin, 2 mg/mL κ -casein. No precipitate was observed.
- Figure 14:** illustrates loss of colloidal stability of the nanocapsules in the presence of calcium chloride and precipitation in the presence of chymosin as measured by A) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and B) size exclusion chromatography (SEC). The size exclusion chromatography picture illustrates the partial aggregation of nanocapsules in presence of calcium and their complete aggregation in the presence of renneting enzyme. The SDS-PAGE results confirm the elimination of the soluble lactoferrin in the fraction corresponding to nanocapsules upon successive addition of calcium and renneting enzyme. Data also shows the coexistence of casein with lactoferrin in the nanocapsules. The figure illustrates the principle of nanocapsule de-stabilization: Colloidal aggregation takes place in presence of calcium and complete precipitation only takes place upon addition of renneting enzyme. The size exclusion chromatography picture shows the partial aggregation of nanocapsules in presence of calcium and their complete aggregation in the presence of renneting enzyme. The SDS-PAGE picture confirms that lactoferrin is eliminated from the soluble fraction upon successive addition of calcium and renneting enzyme to nanocapsules. This picture also confirms the coexistence of κ -casein with lactoferrin in the nanocapsules.
- Concentrations used are: 2 mg/mL lactoferrin, 2 mg/mL κ -casein in 20 mM imidazole, 20 mM NaCl buffer pH 6.5, 10 mg/mL calcium chloride and 0.4 U/mL enzyme.

Figure 15: shows A) DLS data showing a comparison of nanocapsules formulated with dairy casein mixture and nanocapsules formulated with purified dairy κ -casein.

B) Colloidal stability of the nanocapsules formulated with dairy casein mixture in the presence of calcium chloride and chymosin, as observed by SDS-PAGE analysis on the supernatant after the enzymatic reaction and a centrifugation step. When nanocapsules are prepared with a full casein powder instead of purified κ -casein, κ -casein is not functional anymore as the nanocapsules do not loose colloidal stability in the presence of calcium chloride or chymosin and remain in solution. Concentrations used are: 2 mg/mL lactoferrin, 2 mg/mL dairy casein in 20 mM imidazole, 20 mM NaCl, 5.5 mM NaOH buffer pH 6.5, 10 mg/mL calcium chloride and 0.4 U/mL enzyme.

Figure 16: illustrate a demonstration of protective effect of lactoferrin during in vitro digestion. A+B) Scheme of experiment mimicking the gastric phase and the intestinal phase. A) The protein cargo, lactoferrin, is protected against proteolytic degradation during in vitro digestion. Nanocapsules were incubated with a gastric phase followed by an intestinal phase using standard protocols. B) A control was done using free lactoferrin (no nanocapsules added). C) SDS-PAGE show that proteolytic degradation occurs completely in the first incubation step with pepsin only for free lactoferrin whereas a significant fraction of lactoferrin remains intact even after pepsin and pancreatin incubation when added inside the nanocapsules. The SDS-PAGE results measured on the supernatant and precipitate show that lactoferrin survives both the gastric phase incubation and incubation in the presence of intestinal proteolytic enzymes and remain intact. This is not the case if no capsules are present in the lactoferrin control.

Figure 17: illustrate the result from a measurement of encapsulation efficiency for lactoferrin in curd. B) Scheme of Lactoferrin delivery to cheese mass thanks to encapsulation. A) SDS-PAGE of supernatant (whey) shows that 49% of the

fortified lactoferrin is present in the whey when added inside the capsules while 80% remain in the whey if lactoferrin is added in free form (no capsules added).

A) illustrates the result from a measurement of encapsulation efficiency for lactoferrin in curd, wherein free-lactoferrin is used as a control. Curd is formed and lactoferrin concentration is quantified via SDS PAGE measurements in whey. The results, presented in B), show that 51% vs 20% of added lactoferrin is retained in the curd when added in the nanocapsules vs free in the milk.

Concentrations used are: 2 mg/mL lactoferrin, 2 mg/mL κ -casein in 20 mM imidazole, 20 mM NaCl buffer pH 6.5, added 0.2 mg/mL calcium 5 mg/mL citric acid and 0.2 U/mL enzyme.

Figure 18: shows A) comparison of size between lactoferrin nanocapsule and Vitamin B12 nanocapsules measured by DLS. B) Scheme of nanocapsule comprising Vitamin B12 and picture of Vitamin B12 nanocapsule in solution. C) Cheese fortified with Vitamin B12 in nanocapsules (right) or without Vitamin B12 in nanocapsules (left). The cheese changes color when vitamin B12 nanocapsules were added due to the incorporation of the vitamin in the cheese curd.

Figure 19: illustrates increased stability of milk when fortified by nanocapsules as demonstrated by A) visual inspection, B) particle size analyser and C) SDS-PAGE. Milk is stable against aggregation or proteolytic degradation at least up to 12 weeks as compared with less than 2 weeks in the absence of nanocapsules. Controls were made with free lactoferrin, liposomes, buffer, or κ -casein. Concentrations used are: 2 mg/mL lactoferrin, 2 mg/mL κ -casein in 20 mM imidazole, 20 mM NaCl buffer pH 6.5. 5 vol % of nanocapsules were added to 3 % fat milk. The temperature of storage was 4°C.

Figure 20: Stability of nanocapsules in apple juice up to 6 days as demonstrated by A) visual inspection, B) particle size analyzer and C) SDS-PAGE. Fortification with nanocapsules make the juice cloudy and produce a sediment that can be resuspended upon shaking. Controls were made with free

lactoferrin or buffer. SDS-PAGE were run on both the precipitate and the supernatant showing that the cargo protein, lactoferrin, remains inside the capsules even in the sediment. The SDS-PAGE also shows that lactoferrin remains intact when formulated inside of the nanocapsules. Concentrations
5 used are: 2 mg/mL lactoferrin, 2 mg/mL κ -casein in 20 mM imidazole, 20 mM NaCl buffer pH 6.5. 5 vol % of nanocapsules were added to apple juice. The temperature of storage was 4 °C.

Figure 21: Stability of oat milk when fortified with nanocapsules as demonstrated by A) visual inspection, B) particle size analyzer and C) SDS-PAGE. Similar stability was observed at 9 days of storage at 4 °C for oat milk when fortified with free-lactoferrin or nanocapsules. Controls were made with free lactoferrin, buffer, or κ -casein. Concentrations used are: 2 mg/mL lactoferrin, 2 mg/mL κ -casein in 20 mM imidazole, 20 mM NaCl buffer pH 6.5.
15 5 vol % of nanocapsules were added to oat milk.

Figure 22: Soybean milk stability is unchanged when fortified by nanocapsules as demonstrated by A) visual inspection, B) particle size analyzer and C) SDS-PAGE. Controls were made with free lactoferrin, buffer, or κ -casein.
20 Concentrations used are: 2 mg/mL lactoferrin, 2 mg/mL κ -casein in 20 mM imidazole, 20 mM NaCl buffer pH 6.5. 5 vol % of nanocapsules were added to soybean milk. The temperature of storage was 4 °C and samples were monitored for up to 4 weeks.

Figure 23: Yogurt stability is unchanged when fortified by nanocapsules as demonstrated by A) visual inspection, B) particle size analyser and C) SDS-PAGE. Controls were made with free lactoferrin, buffer, liposomes or κ -casein. Concentrations used are: 2 mg/mL lactoferrin, 2 mg/mL κ -casein in 20 mM imidazole, 20 mM NaCl buffer pH 6.5. 5 vol % of nanocapsules were added to
30 yoghurt. The temperature of storage was 4 °C.

Figure 24: shows that plant-based emulsions using bovine κ -casein purified from dairy milk as emulsifier give similar size distribution as emulsions present in milk. This demonstrates that κ -casein emulsification properties are suited for a wide range of food-based systems including dairy analogous. The emulsion
5 was prepared by mixing 3.5 vol% vegetable oil containing 15 mg/mL lecithin with 35 mg/mL κ -casein in 20 mM imidazole, 20 mM NaCl buffer pH 6.5 and subjecting to homogenization.

Figure 25: illustrates stability of the emulsions upon storage at 4 °C (left
10 panels) and 25 °C (right panels) as measured by particle size distribution and visual inspection of the samples. Bovine- κ casein stabilized emulsions are stable upon storage at both 4 and 25 °C at least for up to seven weeks and one week, respectively, as measured by particle size analyzer (top) and visual inspection (bottom). The emulsion in this test was prepared by mixing 3.5 vol%
15 oil containing 15 mg/mL lecithin with 35 mg/mL κ -casein in 20 mM imidazole, 20 mM NaCl buffer pH 6.5.

Figure 26: illustrates stability of the emulsions upon freeze drying and after resuspension in water and storage at 4 °C, as measured by particle size
20 distribution (top) and visual inspection (bottom panels) of the samples. The samples are stable at least after seven weeks of re-suspension and storage at 4 °C. The emulsion was prepared by mixing 3.5 vol% vegetable oil containing 15 mg/mL lecithin with 35 mg/mL κ -casein in 20 mM imidazole, 20 mM NaCl buffer pH 6.5.

25

Figure 27: illustrates that κ -casein in the emulsion surface can be cleaved by chymosin inducing a precipitate that mirrors the dairy cheese curd as shown by A) visual observation and B) SDS-PAGE. The emulsion was prepared by mixing 3.5 vol% oil containing 15 mg/mL lecithin with 35 mg/mL κ -casein in 20
30 mM imidazole, 20 mM NaCl buffer pH 6.5. 0.2 mg/mL calcium chloride, 5 mg/mL citric acid and 0.2 U/mL enzyme were added.

Figure 28: Stability of milk enriched with emulsions of the invention remains unchanged upon storage 4 °C as shown by A) visual inspection, B) particle size distribution and C) SDS-PAGE. Milk enriched with a mixture of caseins loses its stability after 3 weeks. Controls were made with free lecithin in oil, buffer, or κ-casein. 5 vol % of emulsion was added to 3% fat milk.

Description

Definitions

10

As used herein, the singular forms “a”, “an” and “the” include plural referents unless the context clearly states otherwise.

The term “some embodiments” can include one, or more than one
15 embodiment.

As used herein, the term "dispersion" refers to a heterogeneous system having a dispersed phase and a dispersion medium. The dispersed phase may be particles, which for example may be nanocapsules or droplets. Therefore, a
20 dispersion can describe a homogeneous mixture where the dispersed particles will not settle if the solution is left undisturbed for a prolonged period of time or a heterogeneous dispersion of larger particles in a medium where the particles will settle out of the mixture if left undisturbed for a prolonged period of time. Furthermore, a dispersion can describe a heterogeneous
25 mixture where the dispersed particles, for example the nanoparticles or the droplets have at least in one direction a dimension roughly in the nm to μm range. Examples of dispersions herein are suspensions of nanocapsules and emulsions, such as oil-in-water emulsions.

30 As used herein, "emulsion" shall mean a composition, usually liquid, comprising two or more immiscible phases in which a first phase (the "dispersed phase") is dispersed in a second phase (the "continuous phase"). The emulsions of the inventions are preferably oil-in-water emulsions (oil

dispersed in water) comprising oil droplets dispersed in an aqueous phase. Said droplet typically contain a shell and a hydrophobic core.

A nanocapsule according to the invention is a small sphere with a
5 uniform membrane around it (a compartment formed by the membrane). The material inside the nanocapsule (entrapped in the nanocapsule) is referred to as the core, internal phase, or fill, whereas the membrane is sometimes called a shell, coating, or wall. The nanocapsules of the invention typically have diameters between 10 and 200 nanometers. The diameter of the nanocapsule
10 is measured in aqueous phase after homogenization is complete. The diameter of the capsule may change depending on the water activity of the surrounding chemical environment. The nanocapsule can be an artificial compartment whose delimiting borders restrict the exchange of the components enclosed therein. Thus, added value compounds contained in the hydrophilic core of
15 nanocapsules may be completely enclosed by the shell and prohibited from interacting with the surroundings of the nanocapsule as long as the nanocapsule is intact.

The terms "hydrophilic" and "water-soluble" are used interchangeably
20 throughout the description. The terms "hydrophobic" and "oil-soluble" are used interchangeably throughout the description.

The terms "kappa-casein" and " κ -casein" are used interchangeably
25 throughout the description.

An "added value compound" according to the invention may be any
compound, which is desirable to encapsulate and/or transport with the
dispersions of the invention. Examples of added value compounds are food
supplements, nutrients, substances with beneficial nutritional and/or
30 physiological effect or therapeutically active substances.

Dispersion

In a main aspect, the present disclosure concerns a dispersion, said dispersion comprising:

- a) casein, wherein at least 60% of the casein is κ -casein; and
- 5 b) phospholipids.

The invention further relates to methods of producing said dispersions and to methods of producing food products and/or beverage comprising said dispersions.

10

Nanocapsule

In one aspect, the present disclosure concerns a nanocapsule comprising a shell comprising phospholipid and casein, wherein at least 60% of the casein is κ -casein and a hydrophilic core. Thus in some embodiments of
15 this invention, the dispersion is such a nanocapsule or a composition comprising such nanocapsules in an aqueous solution. Nanocapsules can be the dispersed phase of a suspension.

Nanocapsules can be used to deliver hydrophilic added value
20 compounds to hydrophobic environments. This can have high utility in manufacture of cheese or cheese analogues, since the hydrophilic added value compound can be delivered to the cheese or cheese analogue instead of remaining in the whey.

25 Preferably, the nanocapsules are prepared by a method as described in the following chapter "Method of producing nanocapsules".

In some embodiments of this invention, the nanocapsule comprises κ -casein as described in the chapter "casein". The nanocapsules of the invention comprise κ -casein. Whereas the nanocapsules may contain small amounts of
30 other caseins, it is important that the vast majority of caseins in the nanocapsules are κ -casein. In addition, the nanocapsules comprise phospholipids as described in the chapter "phospholipids".

In some embodiments of this invention, the hydrophilic core is enclosed by a hydrophobic shell, wherein the hydrophobic shell contains phospholipids and κ -casein. The hydrophilic core preferably comprises one or more added value compounds, for example hydrophilic added value compounds, which
5 may be any of the added value compounds described in the chapter "Added value compounds". In general, the hydrophobic shell substantially encloses or encapsulates the hydrophilic core.

Emulsions

10 Emulsions can be used to deliver hydrophobic added value compounds to hydrophilic environments. Furthermore, emulsions of this invention can be used to formulate dairy analogues with increased stability of said food products or beverages, and thus do not necessarily comprise any added value compound.

15

In one aspect, the present disclosure concerns an emulsion comprising oil in water droplets comprising a shell comprising casein, wherein at least 60% of the casein is κ -casein and a hydrophobic core. Preferably, the shell further comprises phospholipids. Thus, in some embodiments of this invention, the
20 dispersion is an emulsion comprising oil in water droplets comprising a shell comprising casein, wherein at least 60% of the casein is κ -casein and a hydrophobic core.

The emulsions are preferably prepared by a method as described in the following chapter "Method of producing emulsions".

25 The emulsion comprises κ -casein, preferably as described in the chapter " κ -casein". In addition, the emulsion comprises phospholipids, for example as described in the chapter "phospholipids".

In some embodiments of this invention, the hydrophobic core comprises phospholipids mixed with oil. In some embodiments of this invention, other
30 emulsifiers than phospholipids can be mixed with oil. In addition, the hydrophobic core may comprise one or more added value compound, such as any of the hydrophobic added value compounds described in the chapter "Added value compounds".

K-casein

Casein is a family of related phosphoproteins (α S1, α S2, β , κ) that are commonly found in mammalian milk, constituting about 80% of the proteins in
5 cow's milk. Casein is a mix of the different phosphoproteins α S1-, α S2-, β -, and κ -casein, which naturally occur in bovine casein in a percentage ratio of approximately 40:10:40:10. κ -casein is split by chymosin as found in rennet into an insoluble peptide and water-soluble glycomacropeptide. Interestingly, the present invention discloses that dispersions comprising a high percentage of
10 κ -casein, in contrast to casein mix, lose the colloidal stability in the presence of calcium chloride and precipitate in the presence of chymosin. This is rendering the dispersions particularly useful for production of cheese or cheese analogues.

15 In some embodiments of this invention, the κ -casein is purified from milk, such as from bovine milk, goat milk, sheep milk, camel milk, donkey milk, horse milk, reindeer milk, yak milk or human milk.

In some embodiments of this invention, the κ -casein is recombinant κ -casein. As used herein "recombinant" refers to said κ -casein being produced
20 by recombinant methods. Typically, recombinant κ -casein is produced by a host comprising a heterologous nucleic acid encoding κ -casein. Said heterologous nucleic acid may be comprised within a vector. Thus, recombinant host cells express genes that are not found in identical form within the native (non-recombinant) form of the cell. Thus, in some embodiments of
25 this invention, the κ -casein is recombinant κ -casein produced by a host cell or host organism comprising a heterologous nucleic acid encoding κ -casein.

In some embodiments of this invention, the host cell or host organism is yeast, bacteria, fungi, algae, plants, insect or mammalian. In some
embodiments of this invention, the host cell is a bacterial cell, such as *E. coli*
30 or *B. subtilis*.

In some embodiments of this invention the host organism is an algae, such as *C. reinhardtii* or *C. vulgaris*.

In some embodiments of this invention, the host cell is a yeast cell, such as a yeast cell of the genus *Saccharomyces* or *Pichia*.

In some embodiments of this invention, the host cells are transfected, transformed or transduced with a nucleic acid encoding κ -casein or a functional
5 homologue of said κ -casein.

In some embodiments of this invention, the κ -casein is a κ -casein of SEQ ID NO: 1 or a functional homologue of said κ -casein sharing at least 70%, such as at least 80%, for example at least 90%, such as at least 95% sequence identity therewith. In some embodiments of this invention, the κ -casein is a κ -
10 casein of SEQ ID NO: 2 or a functional homologue of said κ -casein sharing at least 70%, such as at least 80%, for example at least 90%, such as at least 95% sequence identity therewith. In some embodiments of this invention, the κ -casein is a κ -casein of SEQ ID NO: 3 or a functional homologue of said κ -casein sharing at least 70%, such as at least 80%, for example at least 90%,
15 such as at least 95% sequence identity therewith.

The term "functional homologue" of an amino acid sequence, refers to a polypeptide comprising said amino acid sequence with the proviso that one or more amino acids are substituted, deleted, added, and/or inserted, and which
20 polypeptide has (qualitatively) the same functionality. Preferably, a functional homologue shares at least at least 70% sequence identity, preferably at least 80%, preferably at least 85% sequence identity, preferably at least 90% sequence identity, preferably at least 95% sequence identity, more preferred at least 98% sequence identity to said amino acid sequence.

25 The term "sequence identity" as used herein describes the relatedness between two amino acid sequences or between two nucleotide sequences, i.e. a candidate sequence (e.g. a mutant sequence) and a reference sequence (such as a wild type sequence) based on their pairwise alignment. For purposes of the present invention, the sequence identity between two amino
30 acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, J. Mol. Biol. 48: 443- 453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice et al., 2000, Trends Genet.

16:276-277,), preferably version 5.0.0 or later (available at https://www.ebi.ac.uk/Tools/psa/emboss_needle/). The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of 30 BLOSUM62) substitution matrix. The output of Needle
5 labeled "longest identity" (obtained using the -nobrief option) is used as the percent identity and is calculated as follows:

$$(\text{Identical Residues} \times 100) / (\text{Length of Alignment} - \text{Total Number of Gaps in Alignment})$$

The Needleman-Wunsch algorithm is also used to determine whether a
10 given amino acid in a sequence other than the reference sequence corresponds to a given position in a reference sequence. For purposes of the present invention, the sequence identity between two nucleotide sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, supra) as implemented in the Needle program of the EMBOSS package
15 (EMBOSS: The European Molecular Biology Open Software Suite, Rice et al., 2000, Trends Genet. 16: 276-277), preferably version 5.0.0 or later. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the DNAFULL (EMBOSS version of NCBI NUC4.4) substitution matrix. The output of Needle labeled "longest identity" (obtained using the -nobrief option)
20 is used as the percent identity and is calculated as follows:

$$(\text{Identical Deoxyribonucleotides} \times 100) / (\text{Length of Alignment} - \text{Total Number of Gaps in Alignment}).$$

In some embodiments of this invention, at least 70%, such as at least
25 80%, such as at least 90% of the caseins in the dispersion are κ -caseins. In some embodiments of this invention, at most 40%, such as at most 30%, such as at most 20%, such as at most 10%, such as at most 5%, such as at most 1%, such as at most 0,1 % of the caseins in the dispersion are α S1-casein. In some embodiments of this invention, at most 40%, such as at most 30%, such
30 as at most 20%, such as at most 10%, such as at most 5%, such as at most 1%, such as at most 0,1 % of the caseins in the dispersion are α S2-casein. In some embodiments of this invention, at most 40%, such as at most 30%, such as at most 20%, such as at most 10%, such as at most 5%, such as at most

1%, such as at most 0,1 % of the caseins in the dispersion are β -casein. In some embodiments of this invention, at most 40%, such as at most 30%, such as at most 20%, such as at most 10%, such as at most 5%, such as at most 1%, such as at most 0,1 % of the caseins in the dispersion are α S1-casein, α S2-casein and β -casein. In some embodiments of this invention, the casein of the dispersion does not comprise α S1-casein, α S2-casein and/or β -casein. In some embodiments of this invention, the casein of the dispersion does not comprise any of α S1-casein, α S2-casein and β -casein.

10 Apart from phospholipid, the shell of the nanocapsule comprises kappa-casein, or κ -casein, which is a monomeric milk protein that belongs to the group caseins. The κ -casein may comprise animal-free casein, recombinant casein, and milk casein. The milk casein may originate from dairy milk.

Caseins are a family of phosphoproteins that form soluble aggregates known as "casein micelles" in milk. In these casein micelles, the κ -casein is located primarily on the surface, stabilizing the micelle structure.

An advantage with κ -casein in the shell of the nanocapsule is its ability, prior to addition of renneting enzymes, to stabilize the nanocapsule in the milk and to destabilize the nanocapsule in the milk after addition of renneting enzymes. The renneting enzymes cut the hydrophilic parts of the κ -casein, causing the nanocapsules to coagulate and be a part of the curd formation. Colloidal aggregation may take place in presence of calcium and complete precipitation may take place upon addition of the renneting enzyme.

The nanocapsule consists of shell and a space in which at least one water-soluble added value compound, such as a nutrient, is placed. The water-soluble added value compound may comprise a compound selected from the group consisting of a functional protein, a digestive enzyme, an antioxidant, and a vitamin.

30 **Phospholipids**

The dispersions of the invention comprise phospholipids. Many different phospholipids may be comprised within the dispersions of the invention, however, preferred phospholipids are lecithins.

The term Lecithin as used herein describes amphiphilic substances, comprising a mixture of phospholipids. Lecithin can be extracted chemically from natural sources using solvents such as hexane, ethanol, acetone, petroleum ether or benzene. Common sources include egg yolk, marine foods, soybeans, milk, rapeseed, cottonseed, and sunflower oil. In some
5 embodiments of this invention, the phospholipid is a lecithin, which is pure or food grade lecithin.

The term "food grade", as used herein, means and includes, lecithin that
10 is commonly consumed as a food or employed as a food additive. For the purpose of this invention, the term food grade can be understood as materials that are approved by the Regulatory Authorities for use in pharmaceutical, food, nutritional, and/or dietetic applications. The term "pure grade", as used herein, means and includes, lecithin that is purified, but not pure enough to be offered
15 for food, drug, or medicinal use of any kind.

In some embodiments of this invention, the phospholipids are one or more phospholipids selected from the group consisting of synthetic phospholipids, animal-based phospholipids and plant-based phospholipids. In
20 some embodiments of this invention, the phospholipids are a mixture of glycerophospholipids, for example phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, or phosphatidic acid. In some embodiments of this invention, the phospholipids or the lecithin are derived from one or more source selected from the group
25 consisting of egg yolk, marine foods, soybeans, milk, rapeseed, cottonseed, and sunflower oil. In some embodiments of this invention, the phospholipids comprise or consist of lecithin, for example dihexanoyl-L-alpha-lecithin, dioctanoyl-L-alpha-lecithin, didecanoyl-L-alpha-lecithin, didodecanoyl-L-alpha-lecithin, ditetradecanoyl-L-alpha-lecithin, dihexadecanoyl-L-alpha-lecithin,
30 dioctadecanoyl-L-alpha-lecithin, dioleoyl-L-alpha-lecithin, dilinoleoyl-L-alpha-lecithin, or alpha-palmitol. In some embodiments of this invention, the phospholipids are acetone-insoluble phosphatides.

An advantage with phospholipids in the shell is the self-assembling properties of phospholipids. Phospholipids are amphiphilic lipids with hydrophobic fatty acid chains and hydrophilic moieties. The phospholipid may comprise synthetic phospholipid, animal-based phospholipid and/or plant-based phospholipid.

Lecithin is an example of phospholipids. Lecithin may be animal based or plant based. Non-limiting examples of animal lecithin are egg yolk lecithin and milk lecithin. Non-limiting examples of plant based lecithin are soybean lecithin, corn lecithin, rapeseed lecithin, sunflower lecithin, cotton seed lecithin and grain lecithin.

The lecithin may be pure lecithin or food grade lecithin.

Added Value compound

The dispersions of this invention can comprise an added value compound, but can also show a technical effect such as increasing the stability of food products or beverages without any added value compound.

Added value compounds can be either hydrophilic or hydrophobic. Typically, nanocapsules may comprise hydrophilic added value compounds, whereas emulsions comprise hydrophobic added value compounds. Therefore, the dispersion of this invention can enable the encapsulation and/or delivery of a broad spectrum of different compounds to beverages or food products, such as cheese or yoghurt.

The added value compound may any compound, which is beneficial to add to a food product or a beverage. for example, the added value compound may be a food supplement, a nutrient, substances with beneficial nutritional and/or physiological effect or therapeutically active substances.

The added value compound may for example be antibodies or active fragments thereof, peptides, lipids, protein drugs, protein conjugate drugs, enzymes, oligonucleotides, ribozymes or genetic material.

In some embodiments of this invention, the hydrophilic core comprises a water-soluble added value compound. In some embodiments of this

invention, the hydrophilic core is a hydrophilic core as described in the chapter "nanocapsules".

In some embodiments of this invention, the hydrophobic core comprises an oil-soluble added value compound. In some embodiments of this invention,
5 the hydrophobic core is a hydrophobic core as described in the chapter "emulsions".

In some embodiments of this invention, at least one added value compound is a food supplement.

The term "food supplement" in the context of the present invention is
10 interchangeable with the terms food additive, a dietary supplement and nutritional supplement. The term can be understood in a broad sense as any ingestible ingredient capable of supplementing the user's diet. In some embodiments of this invention, the food supplement can be understood as a product to achieve specific benefits. Examples of the benefits include, but are
15 not limited to adding β -galactosidase to milk products to provide milk products for lactose intolerant subjects, adding iron carrying proteins to milk or plant-based products to supplement the diet of vegetarians and vegans or adding vitamins to food products to support the immune system.

20 Thus, in some embodiments of this invention, at least one added value compound is a vitamin, such as vitamin A, vitamin B, vitamin C, vitamin D, vitamin E or vitamin K. In some embodiments of this invention, at least one added value compound is an antioxidant, such as vitamin A, vitamin C, vitamin E, xanthophylls, such as zeaxanthin or lutein, polyphenols or flavonoids such
25 as resveratrol or quercetin. In some embodiments of this invention, at least one added value compound is a protein, such as an iron carrying protein, such as hemoglobin or lactoferrin. In some embodiments of this invention, at least one added-value compound is an enzyme, such as amylase or β -galactosidase. In some embodiments of this invention, at least one added value compound is a
30 fatty acid, such as omega 3 fatty acid or omega 6 fatty acid.

In some embodiments of this invention, at least one added value compound is a therapeutically active substance, such as low molecular weight

therapeutic agents commonly referred to as small molecule drugs or a biologic therapeutically active substance. Thus the added value compound may be antibiotics, peptides and/or hormones. The added value compound may also be antibodies or active fragments thereof, peptides, protein drugs, or protein
5 conjugate drugs.

Examples of hydrophilic added value compounds include but are not limited to water soluble vitamins such as Vitamin C and B, iron carrying proteins such as hemoglobin and lactoferrin, enzymes such as amylase and β -
10 galactosidase, water soluble antioxidants, such as polyphenols and flavonoids and therapeutical components and drugs.

Examples of the hydrophobic added value compounds include, but are not limited to oil soluble vitamins such as Vitamin A, D, and K, antioxidants,
15 such as Vitamin A, vitamin E and xanthophylls, omega 3 and 6 fatty acids, other kinds of essential fats and therapeutical components and drugs.

Non-limiting examples of functional proteins are those for iron supplementation, such as hemoglobin or lactoferrin. Cheese fortification with
20 iron salts, to increase the amount of iron in the food product, is known. However, if iron salts would be used, very high doses would be required because iron has low bioavailability during digestion, which further leads to digestive problems. The iron salts also tend to add a metallic flavor and unwanted color to the final product. This is avoided by the use of lactoferrin.
25 Since iron is incorporated in a metabolizable way, i.e. bound to a protein that favors the uptake, there is no need to use high iron quantities to fortify the cheese. This allows the fortification of cheese in a way that has little impact on its quality, in particular its taste.

The digestive enzyme may give the cheese an added functionality, apart
30 from enhancing its nutritional properties. The digestive enzyme may be beta-galactosidase or amylase. Beta-galactosidase metabolizes lactose. Adding beta-galactosidase to the cheese is thus beneficial for persons who suffer from

lactose intolerance. Amylase breaks complex sugars. The vitamin may be Vitamin B complex. The antioxidant may be glutathione.

The water-soluble added value compound may further comprise a mineral, such as magnesium salts.

5

Method of producing nanocapsules

In one aspect, the present disclosure concerns a method of producing the nanocapsules as described herein, wherein the method comprises the following steps:

10

- a) preparing a lipid film;
- b) preparing a hydrophilic solution comprising casein, wherein at least 60% of the casein is κ -casein and optionally an added value compound;
- c) solubilizing said lipid film prepared in step a) in the solution prepared in step b); and
- d) obtaining a solution containing the nanocapsules.

15

As used herein, "lipid film" refers to lipid in a film form. As used herein, "lipid" refers to a variety of organic compounds. Typically, the lipids include long chain fatty acids and derivatives or analogs thereof. Lipids are, for example, 1) simple lipid (which is ester of fatty acid and an alcohol and may also be referred to as neutral lipid; for example, fat and oil (triacylglycerol), wax (fatty acid ester of higher alcohol), sterolester, fatty acid esters of vitamins, and the like); 2) complex lipid (compounds which have ester bond or amide bond and have a polar group such as phosphoric acid, saccharide, sulfuric acid, amine and the like besides fatty acid and alcohol, including glycerophospholipid, sphingophospholipid, glyceroglycolipid, sphingoglycolipid, lipid having C-P bond, sulfolipid, and the like); 3) derived lipid (compounds produced by hydrolysis of simple lipid and complex lipid which are fat-soluble, including fatty acid, higher alcohol, fat-soluble vitamins, steroid, hydrocarbon, and the like), but not limited to these. In the present invention, any lipid can be used, however, preferably lecithin is used to prepare the lipid film of step a).

25

30

In some embodiments of this invention, the lipid film of step a) is prepared by dissolving phospholipids, for example lecithin in an organic solvent, and subsequently removing said organic solvent, for example evaporating the organic solvent. Thus, the lipid film may essentially consist of
5 phospholipids.

In some embodiments of this invention, the organic solvent in step a) is chloroform or hexane.

In some embodiments of this invention, the lipid film in step a) is prepared by dissolving lecithin.
10

As used herein "hydrophilic" means water soluble. Hydrophilic components are those which are soluble in water at 25 °C at a concentration of 1 weight part hydrophilic component to 9 weight parts water. In general, the hydrophilic solution of step b) is prepared by a method comprising
15 resuspending κ -casein in an aqueous buffer.

In some embodiments of this invention, the hydrophilic solution of step b) comprises an added value compound. The added value compound can be an added value compound as described in the chapter "added value compound". In such embodiments of the invention, the solution in step b) may
20 be prepared by resuspending an added value compound in a buffer and mixing said buffer with a solution comprising said κ -casein.

In some embodiments of this invention, the concentration of κ -casein in the aqueous solution of step b) is at least 0.5 mg/ml, such as at least 1 mg/ml,
25 such as at least 1.5 mg/ml, such as at least 2 mg/ml, such as at least 2.5 mg/ml, such as at least 3 mg/ml, such as at least 4 mg/ml, such as at least 5 mg/ml.

In some embodiments of this invention, the concentration of κ -casein in the aqueous solution of step b) is at most 20 mg/ml, such as at most 10 mg/ml, such as at most 8 mg/ml, such as at most 6 mg/ml, such as at most 4 mg/ml,
30 such as at most 2 mg/ml, such as at most 1 mg/ml.

In some embodiments of this invention, the concentration of κ -casein in the aqueous solution of step b) is between 1 mg/ml and 10 mg/ml.

In some embodiments of this invention, in step c) the solubilization of the lipid film in the hydrophilic solution is achieved by mixing.

In some embodiments of this invention, in step d) of the method the solution comprises phospholipids at a concentration of at least 0.1 mg/ml, such as at least 0.5 mg/ml, such as at least 1 mg/ml, such as at least 2 mg/ml, such as at least 5 mg/ml, such as at least 8 mg/ml, such as at least 10 mg/ml, such as at least 10 mg/ml of the total solution.

In some embodiments of this invention, in step d) of the method the solution comprises phospholipids at a concentration of at most 20 mg/ml, such as at most 10 mg/ml, such as at most 8 mg/ml, such as at most 5 mg/ml, such as at most 2 mg/ml, such as at most 1 mg/ml, such as at most 0.5 mg/ml, such as at most 0.1 mg/ml of the total solution. In some embodiments of this invention, in step d) of the method the solution comprises phospholipids at a concentration of between 5 mg/ml and 10 mg/ml.

15

In some embodiments of this invention, in step c) of the method the lipid film is solubilized by mixing the lipid film of step a) with the solution of step b), wherein the mixing is performed by one or more of vortexing, bath sonication, tip sonication and/or homogenization.

20

The person skilled in the art will appreciate that these mixing methods are commonly used method in the field. Furthermore, the person skilled in the art will appreciate that other methods of mixing the lipid film and the hydrophilic solution can be used.

25

In some embodiments of this invention, in step d) of the method a solution containing nanocapsules comprising a shell made of phospholipid and κ -casein and a core comprising at least one water-soluble added value compound is obtained.

In some embodiments of this invention, the nanocapsules may be further purified, for example via size exclusion chromatography. Thus, the nanocapsules can be eluted from the column at a flow rate of at least 0.1 ml/min, such as at least 0.25 ml/min, such as at least 0.5 ml/min or at a flow rate of at most 1 ml/min, such as at most 0.75 ml/min, such as at most 0.5

30

ml/min. The nanocapsules can be eluted in a buffer comprising sodium chloride and imidazole and/or Bis-Tris.

The phospholipid is preferably as described in chapter "phospholipids".

5

The added value compound is preferably as described in chapter "added value compound".

In some embodiments of this invention, the hydrophilic solution of step
10 b) comprises the added value compound at a concentration of at least 1 mg/ml, such as at least 1.5 mg/ml, such as at least 2 mg/ml, such as at least 3 mg/ml, such as at least 4 mg/ml, such as at least 5 mg/ml, such as at least 6 mg/ml.

In some embodiments of this invention, the hydrophilic solution of step
b) comprises the added value compound at a concentration of at most 10
15 mg/ml, such as at most 8 mg/ml, such as at most 6 mg/ml, such as at most 5 mg/ml, such as at most 4 mg/ml, such as at most 3 mg/ml, such as at most 2 mg/ml, such as at most 1 mg/ml total protein.

In some embodiments of this invention, the hydrophilic solution of step
b) comprises the added value compound at a concentration between 1 mg/ml
20 and 6 mg/ml total protein.

κ -casein is preferably as defined in chapter " κ -casein"

In some embodiments of this invention, the solution in step b) of the
25 method comprises a buffer, for example a buffer comprising sodium chloride and imidazole and/or Bis-Tris. The buffer can have a pH of at least 5, such as at least 5.5, such as at least 6, such as at least 6.5 or a pH of at most 7, such as at most 6.5, such as at most 6, such as at most 5.5, such as at most In some
embodiments of this invention, the buffer has a pH of between 5 and 6.5. The
30 sodium chloride concentration in the buffer can be at least 20 mM, such as at least 40 mM, such as at least 60 mM, such as at least 80mM, such as at least 100 mM, such as at least 120 mM, such as at least 140 mM, such as at least 150 mM or at most 150 mM, such as at most 140 mM, such as at most 120

mM, such as at most 100 mM, such as at most 80 mM, such as at most 60 mM, such as at most 40 mM, such as at most 20 mM. The imidazole concentration in the buffer can be at least 10 mM, such as at least 20 mM, such as at least 30 mM, such as at least 40 mM, or at most 40 mM, such as at most 30 mM, such as at most 20 mM, such as at most 10 mM. The buffer can also comprise Bis-Tris instead of imidazole. The Bis-Tris concentration in the buffer can be at least 10 mM, such as at least 20 mM, such as at least 30 mM, such as at least 40 mM or at most 40 mM, such as at most 30 mM, such as at most 20 mM, such as at most 10 mM.

10

Method of producing emulsions

In one aspect, the present disclosure concerns a method of producing the emulsion as described herein, wherein the method comprises the following steps:

- 15 a) providing a hydrophobic solution, preferably comprising an oil;
 b) preparing a hydrophilic solution comprising casein, wherein at least 60% of the casein is κ -casein;
 c) Emulsifying said hydrophobic solution with said casein solution;
 d) thereby obtaining an emulsion.

20

Preferably, the hydrophobic solution in step a) comprises oil and a phospholipid, such as lecithin.

The oil may be any oil, preferably a food grade oil. For example, the oil may be olive oil, rapeseed oil, soybean oil, sunflower oil or corn oil.

- 25 In some embodiments of this invention, the concentration of the oil is at least 0.5 vol%, such as at least 5 vol%, such as at least 10 vol%, such as at least 15 vol%, such as at least 20 vol% of the hydrophobic solution.

- In some embodiments of this invention, the concentration of the oil is at most 20 vol%, such as at most 15 vol%, such as at most 10 vol%, such as most
30 5 vol% of the hydrophobic solution.

In some embodiments of this invention, the concentration of the oil is between 0.5 vol% and 10 vol% of the hydrophobic solution.

In some embodiments, the concentration of the phospholipids in the hydrophobic solution in step a) of the method of producing emulsions is at least 1 mg/ml, such as at least 5 mg/ml, such as at least 10 mg/ml, such as at least 15 mg/ml. In some embodiments, the concentration of the phospholipids in the hydrophobic solution in step a) of the method of producing emulsions is at most 20 mg/ml, such as at most 15 mg/ml, such as at most 10 mg/ml, such as at most 5 mg/ml. In some embodiments, the concentration of the phospholipids in the hydrophobic solution in step a) of the method of producing emulsions is between 10 mg/ml and 15 mg/ml.

10

In some embodiments of this invention, the hydrophobic solution of step a) comprises an added value compound.

In some embodiments of this invention, the concentration of κ -casein in the solution of step b) is at least 10 mg/ml, such as at least 15 mg/ml, such as at least 20 mg/ml, such as at least 25 mg/ml, such as at least 30 mg/ml, such as at least 35 mg/ml.

In some embodiments of this invention, the concentration of κ -casein in the solution of step b) is at most 40 mg/ml, such as at most 35 mg/ml, such as at most 30 mg/ml, such as at most 25 mg/ml, such as at most 20 mg/ml, such as at most 15 mg/ml, such as at most 10 mg/ml.

In some embodiments of this invention, the concentration of κ -casein in the solution of step b) is between 20 mg/ml and 40 mg/ml.

25 In step d) of the method an emulsion is preferably obtained.

In some embodiments of this invention, in step c) of the method the emulsifying is achieved by mixing. In some embodiments of this invention, the mixing is performed by homogenization.

30 "Homogenization," as the term is used herein, refers to any process for altering particle or droplet size (e.g., reducing size and/or creating size uniformity) in a fluid under conditions of pressure, shear, and/or stress. The term "homogenization" is intended to include the many and varied

homogenization processes that involve the use of ultrasonic, pressure, and/or mechanical forces to homogenize a fluid. Examples of such homogenization techniques include, but are not limited to, two-stage homogenization, high-pressure homogenization (also known as micronization), very high pressure
5 homogenization (VPH), rotator-stator homogenization, blade homogenization, high shear mixers, sonication, high shear impellers, milling, and the like.

The phospholipid is preferably as described in chapter "phospholipids"

10 The added value compound is preferably as defined in chapter "added value compound".

In some embodiments of this invention, the hydrophobic solution of step a) comprises the added value compound at a concentration of at least 0.1
15 mg/ml, such as at least 0.5 mg/ml, such as at least 1 mg/ml, such as at least 1.5 mg/ml, such as at least 2 mg/ml, such as at least 3 mg/ml, such as at least 4 mg/ml, such as at least 5 mg/ml.

In some embodiments of this invention, the hydrophobic solution of step a) comprises the added value compound at a concentration of at most 20
20 mg/ml, such as at most 10 mg/ml, such as at most 5 mg/ml, such as at most 4 mg/ml, such as at most 3 mg/ml, such as at most 2 mg/ml, such as at most 1 mg/ml, such as at most 0.5 mg/ml.

In some embodiments of this invention, the hydrophobic solution of step a) comprises the added value compound at a concentration between 0.1 mg/ml
25 and 10 mg/ml.

κ-casein is preferably as described in chapter "κ-casein".

In some embodiments of this invention, the solution in step b) comprises
30 a buffer. The buffer can comprise sodium chloride and imidazole and/or Bis-Tris. The buffer can have a pH of at least 5, such as at least 5.5, such as at least 6, such as at least 6.5 or of at most 7, such as at most 6.5, such as at most 6, such as at most 5.5, such as at most 5. The sodium chloride

concentration in the buffer can be at least 20 mM, such as at least 40 mM, such as at least 60 mM, such as at least 80mM, such as at least 100 mM, such as at least 120 mM, such as at least 140 mM, such as at least 150 mM or at most 150 mM, such as at most 140 mM, such as at most 120 mM, such as at most 100 mM, such as at most 80 mM, such as at most 60 mM, such as at most 40 mM, such as at most 20 mM. The imidazole concentration in the buffer can be at least 10 mM, such as at least 20 mM, such as at least 30 mM, such as at least 40 mM or at most 40 mM, such as at most 30 mM, such as at most 20 mM, such as at most 10 mM. The buffer can comprise Bis-Tris instead of imidazole. The Bis-Tris concentration in the buffer can be at least 10 mM, such as at least 20 mM, such as at least 30 mM, such as at least 40 mM or at most 40 mM, such as at most 30 mM, such as at most 20 mM, such as at most 10 mM.

15 **Food products and/or beverages**

The present invention provides dispersions, for example nanocapsules or emulsions, which can be used in production of or as an ingredient in food products and/or beverages. For example, they can be used to fortify food products and/or beverages, to increase the stability of the food products/beverages, to transfer added value compounds to food products/beverages, or to enhance stability of added value compounds in food products or beverages. The dispersions of the invention are particularly useful for delivering added value compounds in vivo, because of their stability under conditions mimicking the gastric environment. Even in conditions where the nanoparticles of the invention sediment, the cargo is in general not released and the nanocapsules can be resuspended by shaking. Thus, the dispersions of the invention, and in particular, the nanocapsules of the invention show high stability under conditions mimicking the gastric environment, and therefore they are believed to be particularly useful for transporting added value compounds to the intestines, thereby enabling increased in vivo accessibility of the added value compounds. The dispersion of the invention, and in particular, the nanocapsules of the invention show a high stability also under conditions mimicking the proteolytic activity of the intestinal environment. However, the

content of the dispersions may still be released in the intestines due to the presence of other components of the intestinal environment e.g. the presence of bile acids.

In addition, the dispersions of the invention and in particular the
5 nanocapsules of the invention have high utility in manufacture of cheese or cheese analogues or mixtures thereof.

In addition, the dispersions of the invention and in particular the nanocapsules of the invention have high utility in manufacture of acidic beverages, such as juice.

10

In some embodiments of this invention, the dispersion when mixed with food products and/or beverages increases the stability of said food products and/or beverages.

The term "stability" as used herein refers to storage stability (e.g.,
15 storage stability at room temperature or at 4 °C). For example, in particular stability may describe the lack of aggregation or proteolytic degradation of said food products and/or beverages.

In some embodiments of this invention, the dispersion when mixed with milk increases the stability of said milk, such as decreases the aggregation and
20 proteolytic degradation. In some embodiments of this invention, the nanocapsules increase the stability of milk up to 12 weeks at 4 °C.

In some embodiments of this invention, the dispersion, the nanocapsules or the emulsion as described herein are used for the transport
25 of added value compound(s).

The term "transport" as used herein describes the ability of the dispersion, to deliver the added value compound into food products and/or beverages. In some embodiments of the invention, hydrophilic added value compounds are transported into a hydrophobic environment. In other
30 embodiments of this invention, hydrophobic added value compounds are transported into a hydrophilic environment. However, hydrophilic added value compounds may also be transported into a hydrophilic environment, and hydrophobic added value compounds may also be transported into a

hydrophobic environment. Furthermore, "transport" can also describe the process of delivering added value compounds to a location of interest in vivo. Since the dispersions or the nanocapsules of the invention have high stability under conditions mimicking the gastric environment, they can be used to
5 transport added value compounds, such as food supplements and/or therapeutically active substrates into the body, without being digested in the stomach.

In some embodiments of this invention, the dispersion, the nanocapsules or the emulsion as described herein are used for enrichment of
10 a food products and/or beverages with an added value compound.

In some embodiments of this invention, the food products and/or beverages are dairy products or dairy alternative analogues or mixtures thereof. In some embodiments, the plant-based dairy alternative analogues are
15 plant-base, such as made of soy or other legumes, almond, cashew, coconut, hemp, quinoa and/or oat.

In some embodiments of this invention, the food products and/or beverages are milk, milk analogues, yoghurt, yoghurt analogues, cheese, cheese analogues, juice or mixtures of any of the aforementioned. The milk
20 analogues can be plant based or a mix of dairy and plant-based milk. The yoghurt analogues can be plant based or a mix of dairy and plant-based yoghurt. The cheese analogues can be plant based or a mix of dairy and plant-based cheese.

25 In some embodiments of this invention, the dispersion, the nanocapsules or the emulsion as described herein are used for increasing the nutritional value in food products, such as in cheese, cheese analogues or mixtures thereof and/or in beverages.

The term "nutritional value" is associated with the quantity of available
30 micronutrients, like iron, vitamins, antioxidants and other food supplements in the food product. In a preferred embodiment of the invention the amount of nutrients in the food product is increased. In this context the term "increased" means an increase of at least 1%, preferably of at least 5%, even more

preferably of at least 10% and most preferably of at least 20% of the food supplement when compared to the food product without added dispersion.

In one aspect, the present disclosure concerns a method of producing a
5 food product and/or beverages, wherein the method comprises the following steps:

- a) Providing a food product, a beverage, food product ingredient or a beverage base;
- b) contacting said food product, beverage, food product ingredient
10 or beverage base with the dispersion, the nanocapsules or the emulsion as described herein,
- c) processing the mixture of step b) into a product or a beverage.

In some embodiments of this invention, the food product or the beverage
15 is as described above.

In some embodiments of this invention, the food product is cheese, a cheese analogue or mixtures thereof.

In some embodiments of this invention, the dispersion, the nanocapsule and/or the emulsion are added in step b) to the food product, beverage, food
20 product ingredient or beverage base at a concentration of at least 3 vol%, such as at least 4 vol%, such as at least 5 vol% of the food product, beverage, food product ingredient or beverage base.

In some embodiments of this invention, the dispersion, the nanocapsule and/or the emulsion are added in step b) to the food product, beverage, food
25 product ingredient or beverage base at a concentration of at most 50 vol%, such as at most 40 vol%, such as at most 30 vol%, such as at most 10 vol%, such as at most 8 vol%, such as at most 6 vol%, such as at most 5 vol% of the food product, beverage, food product ingredient or beverage base.

In some embodiments of this invention, the dispersion, the nanocapsule
30 and/or the emulsion are added in step b) to the food product, beverage, food product ingredient or beverage base at a concentration of 5 vol% of the food product, beverage, food product ingredient or beverage base. The term "beverage base" as used herein means an intermediate beverage product

which, when mixed with an appropriate amount of water or other suitable liquid or semi-liquid and/or a sweetening agent, forms a beverage syrup or alternatively a beverage. The term "food product ingredient" includes a formulation which is or can be added to food products or foodstuffs, for example, as a nutritional supplement. The term food ingredient as used here also refers to formulations which can be used at low levels in a wide variety of products that require stabilizing.

Cheese

The dispersion of this invention has a high utility in manufacture of cheese or cheese analogues or mixtures thereof. Accordingly, whereas this section refers to "cheese", the methods disclosed herein are equally applicable to cheese analogues or mixtures thereof. The cheese analogues can be plant based or a mix of dairy and plant-based cheese. The dispersion of this invention can be used in different methods of cheese making. When added value compounds are delivered using the dispersions or the nanocapsules of the invention during cheese manufacturing, the added value compounds can will typically be delivered to the cheese or cheese analogue, rather than remaining in the whey. In other words, the cheese or cheese analogue will have a higher level of the added value compounds, whereas the waste product, the whey will have a lower level. This is in particular the case for hydrophilic added value compounds.

In one aspect, the present disclosure concerns a method of producing cheese or cheese analogues, wherein the method comprises the following steps:

- a) Providing milk or milk analogue or mixture thereof comprising the dispersion, the nanocapsules or the emulsion as described herein;
- b) processing said milk or milk analogue into cheese.

In a preferred embodiment of this invention, the provided milk in the method of producing cheese in step a) is non-homogenized milk.

In some embodiments of this invention, the milk or milk analogue of step a) of the method of producing cheese or cheese analogue comprises at least 2.5% fat, such as at least 2.7% fat, such as at least 2.9% fat, such as at least 3% fat, such as at least 3.1% fat.

- 5 In some embodiments of this invention, the milk or milk analogue of step a) of the method of producing cheese or cheese analogue comprises at most 4.8% fat, such as at most 4% fat, such as at most 3.5% fat, such as at most 3% fat, such as at most 2.5% fat.

- 10 In some embodiments of this invention, the milk or milk analogue of step a) of the method of producing cheese or cheese analogue comprises between 2.9% and 3.1% fat.

- 15 In some embodiments of this invention, calcium chloride is added in the method of producing cheese or cheese analogue at a concentration of at least 0.2 mg/ml, such as at least 0.5 mg/ml, such as at least 1 mg/ml, such as at least 2 mg/ml, such as at least 5 mg/ml, such as at least 8 mg/ml, such as at least 10 mg/ml.

- 20 In some embodiments of this invention, calcium chloride is added in the method of producing cheese or cheese analogue at a concentration of at most 10 mg/ml, such as at most 8 mg/ml, such as at most 5 mg/ml, such as at most 2 mg/ml, such as at most 1 mg/ml, such as at most 0.5 mg/ml, such as at most 0.2 mg/ml.

- 25 In some embodiments of this invention, calcium chloride is added in the method of producing cheese or cheese analogue at a concentration of between 0.2 mg/ml and 10 mg/ml.

- 30 In some embodiments of this invention, the milk or milk analogue is acidified by adding citric acid in the method of producing cheese or cheese analogue. In some embodiments of this invention, the citric acid is added at a concentration of at least 1 mg/ml, such as at least 2 mg/ml, such as at least 3 mg/ml, such as at least 4 mg/ml, such as at least 5mg/ml. In some embodiments of this invention, the citric acid is added at a concentration of at most 5 mg/ml, such as at most 4 mg/ml, such as at most 3 mg/ml, such as at most 2 mg/ml, such as at most 1 mg/ml.

In some embodiments of this invention, the milk or milk analogue is equilibrated in the method of producing cheese or cheese analogue at a temperature of at least 30 °C, such as at least 31 °C, such as at most 32 °C. In some embodiments of this invention, the milk or milk analogue is equilibrated
5 in the method of producing cheese or cheese analogue at a temperature of at most 34 °C, such as at most 33°C, such as at most 32 °C. In some embodiments of this invention, the milk or milk analogue is equilibrated in the method of producing cheese or cheese analogue at a temperature of 32 °C. In some embodiments of this invention, the milk or milk analogue is equilibrated
10 in the method of producing cheese or cheese analogue for at least 10 minutes, such as at least 15 minutes, such as at least 20 minutes. In some embodiments of this invention, the milk or milk analogue is equilibrated in the method of producing cheese or cheese analogue for at most 40 minutes, such as at most 30 minutes, such as at most 20 minutes. In some embodiments of this
15 invention, the milk or milk analogue is equilibrated in the method of producing cheese or cheese analogue for 20 minutes.

In some embodiments of this invention, the concentration of the chymosin enzyme in the milk or milk analogue is at least 0.05 U/ml, such as at
20 least 0.1 U/ml, such as at least 0.15 U/ml, such as at least 0.2 U/ml. In some embodiments of this invention, the concentration of the chymosin enzyme in the milk or milk analogue is at most 0.4 U/ml, such as at least 0.3 U/ml, such as at least 0.2 U/ml. In some embodiments of this invention, the concentration of the chymosin enzyme in the milk or milk analogue is between 0.2 U/ml and
25 0.4 U/ml.

In some embodiments of this invention, curd is cut during the incubation process.

In some embodiments of this invention, step b) comprises the steps of
30

- Adding calcium chloride to the milk or milk analogue of step a);
- Acidifying the milk or milk analogue;
- Equilibrating the milk or milk analogue;
- Adding chymosin to the milk or milk analogue;

- Incubating the milk or milk analogue to obtain curd;
- Incubating the curd to obtain cheese or cheese analogue; and
- Separating the cheese mass or cheese analogue mass from whey.

5

In some embodiments of this invention, the cheese mass or cheese analogue mass and the whey are separated in step i) by filtration.

10 The present invention also relates to a nanocapsule for encapsulation and delivery of water-soluble added value compounds to cheese. The nanocapsule comprises:

- a shell comprising phospholipid and κ -casein, and
- a core comprising at least one water-soluble added value compound.

15 The present invention is based on the understanding that a nanocapsule which comprises phospholipid and κ -casein in the shell provides a stable container for delivery of added value compounds to cheese, which container destabilizes and coagulates upon addition of a renneting enzyme. The nanocapsules are used to incorporate water-soluble molecules with the
20 objectives to increase the nutritional value of the cheese, and to add a health promoting functionality.

The present inventive concept further relates to a method to produce a nanocapsule for encapsulation and delivery of water-soluble added value compounds to cheese. The method comprising the steps:

25 a) preparing a lipid film by dissolving phospholipids in an organic solvent and evaporating the organic solvent;

b) preparing an aqueous solution comprising at least one water-soluble added value compound;

c) preparing a κ -casein solution,

30 d) solubilizing said lipid film prepared in step a) by adding to and mixing with said solutions prepared in step b), and

e) adding the κ -casein solution obtained in step c) thus obtaining a solution containing nanocapsules comprising a shell made of phospholipid and

κ -casein and a core comprising at least one water-soluble added value compound,

wherein step e) occurs simultaneously with or after step d).

The solvent used in step a) may be an organic solvent, such as
5 chloroform or hexane.

The evaporation of step a) may be conducted under vacuum by use of desiccator or a plate evaporator. It should be noted that any type of solvent evaporator may be used for evaporation of the organic solvent.

In the case where step e) occurs simultaneously with step d), a protein
10 solution comprising at least one water-soluble added value compound and κ -casein is prepared. The protein solution may typically be an aqueous protein solution. This protein solution may be obtained by mixing a solution comprising at least one water-soluble added value compound with a κ -casein solution. The solution comprising the water-soluble added value compound and the κ -casein
15 solution, respectively, may be aqueous solutions.

In case the water-soluble compound is a protein, the protein concentration of step b) may be up to 10 mg/mL, preferably from 1 to 6 mg/mL.

The concentration of κ -casein in the κ -casein solution of step c) may be up to 20 mg/mL, preferably from 1 to 10 mg/mL.

20 The concentration of phospholipids in the solubilized lipid film of step d) may be from 0.1 to 20 mg/mL, preferably from 5 to 10 mg/mL.

The preparation in step b) and/or step c) may comprise the steps of:

a') providing a powder comprising κ -casein and/or at least one water-soluble added value compound, and
25 b') suspending said powder in a buffer solution containing imidazole or Bis-Tris (2-bis(2-hydroxyethyl)amino-2(hydroxymethyl)-1,3-propanediol, bis(2hydroxyethyl)amino-tris(hydroxymethyl)methane, 2,2-bis(hydroxymethyl)-2,2',2''-nitrilotriethanol) and sodium chloride.

The concentration of imidazole or Bis-Tris in the buffer solution may be
30 between 10 and 40 mM. The concentration of sodium chloride in the buffer solution may be between 20 and 150 mM. The buffer solution may have a pH of from 4.5 to 7, preferably from 5.5 to 6.7.

In step d), a solution comprising nanocapsules and excess of κ -caseins and/or phospholipids is obtained. In order to provide nanocapsules of good quality, it is important that mixing is good. To this end, step d) may comprise mixing via vortexing, extrusion, homogenization and/or sonication for
5 solubilization of the lipid film.

The present inventive concept further relates to a method for production of fortified cheese and/or cheese with added functionality. The method comprising the steps of:

- a) providing the nanocapsule according to the inventive concept;
- 10 b) adding the nanocapsule to milk thus forming fortified milk or milk with added functionality;
- c) adding renneting enzymes to the fortified milk or milk with added functionality, thus forming fortified cheese curd and whey;
- d) separating the fortified or functional cheese curd from the whey; and
- 15 e) pressing the fortified or functional cheese curd into cheese.

The process consists of using the self-assembly properties of κ -casein and combining it with the self-assembly properties of phospholipids to create nanocontainers with the capacity to hold e.g. enzymes and other components of high nutritional value. The nanocapsules are added to the milk, which
20 comprises casein micelles, to provide fortified milk or milk with added functionality. After addition of the nanocapsules, renneting enzymes, such as chymosin, are added. Thus, step b) occurs before the step c). When rennet is added, i.e. during the coagulation phase, the soluble part of the κ -casein present in the nanocapsules and in the casein micelles, respectively, is cleaved
25 and a cheese curd is formed. Upon renneting, the nanocapsules thus coagulate together with the casein micelles in the milk and form a cheese curd with incorporated and protected water-soluble nutrients.

It is believed that colloidal aggregation takes place in presence of calcium under an acid condition but full precipitation only takes place upon
30 addition of renneting enzyme.

The milk used in step b) may comprise plant-based milk and/or dairy milk or mixtures thereof. The milk may comprise between 2.5 and 4.8% by

weight, preferably between 2.9 and 3.1% by weight of fat. The milk may be non-homogenized milk.

Typically, 0.05-0.4 Units/mL renneting enzymes may be added to the fortified milk.

5 The method may further comprise the step of:

f) enriching the milk with calcium chloride, wherein the step f) occurs after step b). Typically, 0.2 to 10 mg/mL of calcium chloride may be added to the milk.

The method may further comprise the step of:

10 g) acidifying the milk by adding an acid, such as citric acid, or by adding starter, such as lactic acid bacteria, to the milk, wherein the step g) occurs after step b). Typically, 1 to 5 mg/mL of citric acid may be added to the milk.

Starter is used for production of acids and flavour. The concentration of starter may be 10^4 to 10^8 Colony Forming Unit/mL.

15 The reason why step f) and g), respectively, occur after step b) is that addition of calcium chloride and acidification may induce colloidal aggregation of the casein micelles. If the aggregation takes place before the addition of the nanocapsules, it is believed that the nanocapsules may be poorly incorporated into the cheese curd.

20 The present inventive concept further relates to a fortified cheese and/or a cheese with added functionality obtained by the method. A result of the method may thus be a fortified cheese or a cheese with added-value or added functionality, the cheese being provided with good structure, taste, and visual appearance.

25

Embodiments

The invention may further be defined by the following embodiments:

1. A nanocapsule for encapsulation and delivery of water-soluble added
30 value compounds to cheese, said nanocapsule comprising:
 a shell comprising phospholipid and κ -casein, and
 a core comprising at least one water-soluble added value compound.

2. A nanocapsule according to embodiment 1, wherein said phospholipid comprises synthetic phospholipid, animal-based phospholipid and/or plant-based phospholipid.
- 5 3. A nanocapsule according to embodiment 1 or 2, wherein said κ -casein comprises animal-free casein, recombinant casein, and milk casein.
4. A nanocapsule according to any one of the preceding embodiments, wherein said water-soluble added value compound comprises a compound
10 selected from the group consisting of a functional protein, a digestive enzyme, an antioxidant, and a vitamin.
5. A method to produce a nanocapsule for encapsulation and delivery of water-soluble added value compounds to cheese, said method comprising the
15 steps:
 - a) preparing a lipid film by dissolving phospholipids in an organic solvent and evaporating the organic solvent;
 - b) preparing an aqueous solution comprising at least one water-soluble added value compound;
 - 20 c) preparing a κ -casein solution,
 - d) solubilizing said lipid film prepared in step a) by adding to and mixing with said solutions prepared in step b), and
 - e) adding said κ -casein solution obtained in step c) thus obtaining a solution containing nanocapsules comprising a shell made of phospholipid and
25 κ -casein and a core comprising at least one water-soluble added value compound,
wherein step e) occurs simultaneously with or after step d).
6. A method according to embodiment 5, wherein said solvent used in step
30 a) is an organic solvent, such as chloroform or hexane.
7. A method according to embodiment 5 or 6, wherein said evaporation of step a) is conducted under vacuum by use of a desiccator or a plate evaporator.

8. A method according to any one of embodiment 5 to 7, wherein said preparation in said step b) and/or step c) comprises the steps of:

5 a') providing a powder comprising κ -casein and/or at least one water-soluble added value compound, and

b') suspending said powder in a buffer solution containing imidazole or bis tris (2-bis(2-hydroxyethyl)amino-2(hydroxymethyl)-1,3-propanediol, bis(2hydroxyethyl)amino-tris(hydroxymethyl)methane, 2,2-bis(hydroxymethyl)-2,2',2''-nitrilotriethanol) and sodium chloride.

10

9. A method according to embodiment 8, wherein said buffer solution has a pH of from 4.5 to 7, preferably from 5.5 to 6.7.

10. A method according to any one of embodiment 5 to 9, wherein said step
15 d) comprises mixing via vortexing, homogenization, extrusion and/or sonication for solubilization of the lipid film.

11. A method for production of fortified cheese and/or cheese with added functionality, said method comprising the steps of:

20

a) providing said nanocapsule according to embodiment 1;

b) adding said nanocapsule to milk thus forming fortified milk or milk with added functionality;

c) adding renneting enzymes to said fortified milk or milk with added functionality, thus forming fortified cheese curd and whey;

25

d) separating said fortified or functional cheese curd from said whey; and

e) pressing said fortified or functional cheese curd into cheese.

12. A method according to embodiment 11, wherein said milk comprises plant-based milk and/or dairy milk.

30

13. A method according to embodiment 11 or 12, further comprising the step of:

f) enriching said milk with calcium chloride, wherein said step f) occurs after step b).

14. A method according to any one of embodiment 11 to 13, further comprising the step of:

g) acidifying said milk by adding an acid, such as citric acid, or by adding starter, such as lactic acid bacteria, to said milk, wherein said step g) occurs after step b).

15. A fortified cheese and/or a cheese with added functionality obtained by the method according to any one of embodiments 11 to 14.

Examples

Example 1: Nanocapsules and emulsions can be made using κ -casein recombinant produced in *E. coli*

Aim:

To investigate if recombinant expressed κ -casein can be used to produce nanocapsules and emulsions.

20

Material and Methods:

Production of recombinant κ -casein in *E. coli*:

Expression: A codon optimized sequence encoding κ -casein from donkey of SEQ ID NO:3 (*Equus asinus africanus* – Uniprot accession number F0V6V5, 23 May 2024) was cloned in pET30a plasmid. The recombinant plasmid was used for the expression of the protein in *E. coli*. Bacterial cells were grown at 37 °C in Terrific Broth (12 g/L tryptone, 24 g/L yeast extract, 0.5% glycerol, 89 mM phosphate buffer) supplemented with 30 µg/mL kanamycin. Protein expression was induced by adding 1 mM isopropyl-beta-thiogalactopyranoside (IPTG) when the bacterial cultures reached an optical density at 600 nm of 0.5 - 0.7. The expression of κ -casein was performed at 37 °C for 5 hours. At the end of the expression phase, cells were harvested by

centrifugation and lysed in lysis buffer (20 mM imidazole, pH 6.5, 20 mM NaCl) by sonication (10 % amplitude, 30 min total time, 2 sec on, 2 sec off, on ice).

- Purification: Donkey κ -casein was purified from bacterial cell lysate by cationic exchange chromatography (CIEX) on an Äkta Pure FPLC system equipped with a Hi-Trap SP column (Cytiva). The CIEX method for purification employed a wash/equilibration buffer (also known as buffer A: 20 mM imidazole, pH 6.5, 20 mM NaCl) and an elution buffer (also known as buffer B: 20 mM imidazole, pH 6.5, 1 M NaCl), according to the following steps:
- 10 Column equilibration: 5 column volume (CV) of buffer A at 5 ml/min
 - Loading of bacterial cell lysate on the column: at 2.5 ml/min
 - Washing out unbound sample: 5 CV, at 5 ml/min
 - Elution according to buffer B gradient: from 0 to 100 % of buffer B in 12 CV
 - 15 Column wash: 5 CV 100% buffer B

Donkey κ -casein peak eluted at 60 % of buffer B. Protein purity was analyzed by stain-free SDS-PAGE and protein concentration determined by using a NanoDrop.

20

Preparation of nanocapsules:

- A lipid film was prepared by dissolving pure soy lecithin in hexane. Hexane was evaporated overnight using a desiccator under vacuum. The lipid film was stored at -20 °C until use.
- 25 0.5 mg/mL Lactoferrin was mixed with 0.8 mg/mL recombinant donkey κ -casein. The aqueous solution for resuspension was a buffer containing 20 mM imidazole, 60 mM sodium chloride and pH 6.5.

- The lipid film was solubilized by addition of 10 mg/mL lactoferrin solution to the film. Gentle mixing via vortexing and then tip sonication (Sonifier S-450D, 400 Watts, Branson Ultrasonics, Connecticut, USA) was performed for complete resuspension. The sample was sonicated for 1.5 min.
- 30

Preparation of emulsions:

3.5 vol% olive oil enriched with 15 mg/mL lecithin was mixed with 4 mg/mL recombinant donkey κ -casein. The aqueous solution for casein was a buffer containing 20 mM imidazole, and 60 mM sodium chloride and pH 6.5.

- 5 Emulsions were prepared by T25 digital ULTRA-TURRAX homogenizer (IKA-Werke GmbH & Co. KG, Staufen, Germany). Sample were homogenized 15000 rpm, 10 min, 25 °C.

Results and Conclusion:

- 10 The inventors of the present disclosure have shown that nanocapsules and emulsions can be produced using recombinant κ -casein from donkey (Figure 3).

Example 2: Nanocapsules and emulsions can be made using κ -casein recombinant

- 15 produced in *C. reinhardtii*

Aim:

To investigate if recombinant expressed κ -casein can be used to produce nanocapsules and emulsions.

20

Material and methods:

Production of recombinant κ -casein in *C. reinhardtii*:

- Expression and detection: A codon optimized sequence encoding κ -casein from bovine of SEQ ID NO:2 (Bos taurus– Uniprot accession number
25 P02668, 23 May 2024) was cloned in pChlamy _4 vector, which allows the constitutive expression of the recombinant protein in the intracellular compartment of transformed cells. The recombinant vector was introduced in the expression host – *Chlamydomonas reinhardtii* by electroporation. Cells were grown in Tris Acetate Phosphate medium (also known as TAP medium)
30 supplemented with 2.5 μ g/ml zeocin, at 26 °C with light source set at 50 μ E m⁻² s⁻¹. After 3 days of incubation, recombinant microalgae were harvested, lysed in lysis buffer (20 mM imidazole, pH 6.5, 20 mM NaCl) by sonication (20 % amplitude, 30 min total time, 30 sec on, 30 sec off, on ice), and bovine κ -

casein protein levels were determined by stain-free SDS-PAGE followed by Western blot developed by using a polyclonal rabbit anti-bovine κ -casein primary antibody (AA 111-170, antibodies-online) and a polyclonal HRP-conjugated anti-rabbit secondary antibody (31460, Invitrogen).

5

The emulsion was produced as described in example 1, with the exception that 70 mg/mL algae protein including bovine κ -casein was used instead of donkey κ -casein. Sample were homogenized 15000 rpm, 15 min, 25 °C.

10

Results and Conclusion:

The inventors of the present disclosure have shown that emulsions can be produced using recombinant bovine κ -casein (Figure 4).

15 Example 3: Nanocapsules are a single type of particle

Aim:

To investigate the size of the nanocapsules.

Material and methods:

20 Preparation of nanocapsules

A lipid film was prepared by dissolving pure soy lecithin in hexane. Hexane was evaporated overnight using a desiccator under vacuum. The lipid film was stored at -20 °C until use. 2 mg/mL lactoferrin was mixed with 2 mg/mL κ -casein from bovine milk. The aqueous solution for resuspension was a buffer
25 containing 20 mM imidazole, 20 mM sodium chloride and pH 6.5.

The lipid film was solubilized by addition of 10 mg/ml lactoferrin solution to the film. Gentle mixing via vortexing and then tip sonication (Sonifier S-450D, 400 Watts, Branson Ultrasonics, Connecticut, USA) was performed for
30 complete resuspension. The sample was sonicated.

Dynamic light scattering (DLS):

DLS was performed by using a Zetasizer Ultra (Malvern Panalytical, Ltd., Malvern, UK) using a quartz cuvette (ZEN2112), loaded with a sample volume of 90 µL. All measurements were performed on freshly prepared samples, at 25 °C and data were processed using the software Zetasizer Ultra-Pro ZS
5 Xplorer v1.31. A refractive index (RI) 1.45 for liposomes and 1.33 for water were used to calculate the size.

Results and Conclusion:

The inventors of the present disclosure have shown that nanocapsules
10 are a single type of particle that differ from that of its single components (Figure 8).

Example 4: Nanocapsules made with plant-based purified lecithin

Aim:

15 To investigate if nanocapsules can be made from different sources of lecithin.

Material and methods:

The nanocapsules were prepared as described in example 3, with the
20 exception that 0.2 mg/mL lecithin from either soy or sunflowers were dissolved in chloroform. The lecithin was either foodgrade lecithin or purified lecithin. Furthermore, 1.36 mg/mL κ-casein from bovine milk without encapsulated protein in 25 mM Bis-Tris buffer, 25 mM sodium chloride and pH 6.7 were used. The sample was sonicated at 80 % amplitude, 5 min, 0.5 sec on, 0.5 sec off,
25 25 °C.

DLS experiment was performed as described in example 1.

Results and Conclusion:

30 The inventors of the present disclosure have shown that nanocapsules can be made with plant-based purified lecithin, and also with food graded lecithin from soy lecithin and sunflower lecithin (Figure 9).

Example 5: Stability of nanocapsulesAim:

To investigate the stability of the nanocapsules in different pH and salinity conditions.

5

Material and methods:

The nanocapsules were prepared as described in example 3, with the exception that either 0.5 mg/ml lecithin for the stability test in different pH or 1 mg/mL lecithin for the stability test in different salinity conditions from soy were dissolved in chloroform. Furthermore, 1.36 mg/mL κ -casein from bovine milk without encapsulated protein either 25 mM Bis-Tris buffer, 25 mM sodium chloride and pH 4.6-6.7 were used for the stability test in different pH or 20 mM imidazole buffer, 0-100 mM sodium chloride, pH 6.5 were used for the stability test in different salinity conditions. The sample was sonicated at 80 % amplitude, 5 min, 0.5 sec on, 0.5 sec off, 25 °C.

10
15Results and Conclusion:

The inventors of the present disclosure have shown that the nanocapsules are stable at pH 4.6 to 6.7 and in salinity conditions of between 20 to 100 mM NaCl in imidazole buffer at pH 6.5 (Figure 10).

20

Example 6: Nanocapsule structureAim:

To determine the nanocapsule structure.

25

Material and methods:

Preparation of nanocapsules

30

The nanocapsules were prepared as described in example 3, with the exception that 3 mg/mL pure soy lecithin dissolved in chloroform and 1.36 mg/mL κ -casein from bovine milk without encapsulated protein in 20 mM

imidazole buffer, 60 mM sodium chloride and pH 6.5 was used. The sample was sonicated at 80 % amplitude, 5 min, 0.5 sec on, 0.5 sec off, 25 °C.

Small Angle Xray Scattering (SAXS):

5 Data was collected on a Xeuss 3.0 instrument using 1 mm capillaries. Data was analysed using SASView. SAXS data was fitted using core multi shell model.

Cryogenic-Transmission Electron Microscopy (Cryo-TEM):

10 Cryo-TEM was performed at University of Copenhagen, Denmark, on FEI Tecnai G2 20 TWIN is a 200 kV Transmission Electron Microscope.

Results and Conclusion:

15 The inventors of the present disclosure have shown that the nanocapsules have a core-shell structure, in which the core is water filled and the shell is composed of phospholipids and κ -casein (Figure 11).

Example 7: Stability of the nanocapsules upon storage

20

Aim:

To determine the stability of the nanocapsules upon storage.

Material and methods:

25 The nanocapsules were prepared as described in example 3. DLS was performed as described in example 1.

Results and Conclusion:

30 The inventors of the present disclosure have shown that the nanocapsules are stable for at least up to 38 weeks at 4 °C and 5 weeks at 25 °C (Figure 12).

Example 8: Stability of the nanocapsules upon freeze dryingAim:

To determine the stability of the nanocapsules upon freeze drying

5

Material and methods:

The nanocapsules were prepared as described in example 3. DLS was performed as described in example 1.

10 Results and Conclusion:

The inventors of the present disclosure have shown that the samples are stable after at least 7 weeks of re-suspension and storage at 4 °C (Figure 13).

15 Example 9: Loss of colloidal stability of the nanocapsules in the presence of calcium chloride and precipitation in the present of chymosinAim:

To determine if the nanocapsules are incorporated into the cheese curd.

20

Material and methods:

The nanocapsules were prepared as described in example 3. To test the colloidal stability of the nanocapsules either 10 mg/mL calcium chloride or 10 mg/mL calcium chloride and 0.4 U/mL chymosin was added. DLS was performed as described in example 1. Furthermore, SDS-PAGE was performed.

25

Results:

The colloidal stability of the nanocapsules is decreased in the presence of calcium chloride and the nanocapsule fully precipitate in presence of calcium chloride and chymosin.

30

Conclusion:

The inventors of the present disclosure have shown that the nanocapsules are incorporated into the cheese curd (Figure 14).

Example 10: Comparison of nanocapsules comprising casein mixture with
nanocapsules comprising purified κ -casein

Aim:

To compare nanocapsules comprising casein mixture with nanocapsules comprising κ -casein.

Material and methods:

The nanocapsules were prepared as described in example 3 or in case of the nanocapsules comprising the casein mixture, 2 mg/ml dairy casein in 20 mM imidazole, 20 mM NaCl, 5.5 mM NaOH buffer, pH 6.5 was used. To test the colloidal stability of the nanocapsules either 10 mg/mL calcium chloride or 10 mg/mL calcium chloride and 0.4 U/mL chymosin was added. DLS was performed as described in example 1. Furthermore, SDS-PAGE was performed.

Results:

When nanocapsules are prepared with a full casein powder instead of purified κ -casein, κ -casein is not functional anymore as the nanocapsules do not loose colloidal stability in the presence of calcium chloride or chymosin (Figure 15).

Conclusion:

The inventors of the present disclosure have shown that κ -casein is needed to achieve the effect of the nanocapsules of this invention, thus to loose colloidal stability in the presence of calcium chloride or chymosin.

Example 11: The protein cargo is protected against proteolytic degradation during in vitro digestion

Aim:

To investigate if the protein cargo of the nanocapsules is protected against proteolytic degradation during in vitro digestion.

5 Material and methods:

The nanocapsules were prepared as described in example 3. To mimic the gastric phase, 1.7 mg/mL pepsin was added to the nanocapsules and incubated for 1 hour at 37 °C under shaking at 300 rpm. To mimic the proteolytic activity of the intestinal phase, 0.4 mg/mL pancreatin was added to
10 the gastric phase mixture. The intestinal phase mixture was incubated for 2 hours at 37 °C. Free lactoferrin was used as a control. SDS-PAGE was performed.

Results:

15 SDS-PAGE show that proteolytic degradation occurs completely in the first incubation step with pepsin only for free lactoferrin whereas a significant fraction of lactoferrin remains intact even after pepsin and pancreatin incubation when added inside the nanocapsules (Figure 16).

20 Conclusion:

The inventors of the present disclosure have shown that the protein cargo, lactoferrin, is protected against proteolytic degradation during in vitro digestion, when encapsulated in the nanocapsules.

25 Example 12: Delivery of lactoferrin to cheeseAim:

To investigate if the encapsulation of lactoferrin in the nanocapsules leads to delivery of lactoferrin into cheese mass.

30

Material and methods:

The nanocapsules were prepared as described in example 3.

For enrichment of cheese, the nanocapsules were added to 2.9-3.1% fat non-homogenized milk. Before adding the rennet, the milk was further enriched with 0.2 mg/mL calcium chloride and acidified by adding 5 mg/mL citric acid. Then, the milk was equilibrated at 32 °C for 20 minutes and 0.2 U/mL chymosin enzyme was added. Incubation took place at 32 °C for 15 minutes. The curd was cut, and further incubation takes place at 32 °C for 15 min. During the incubation process, the curd was cut in 5 minutes intervals. The cheese mass was then separated from whey by filtration. The concentration of lactoferrin was determined via SDS-PAGE.

Results:

SDS-PAGE of supernatant (whey) shows that 49% of the fortified lactoferrin is present in the whey when added inside the capsules while 80% does if no capsules are added (Figure 17).

Conclusion:

The inventors of the present disclosure have shown that the encapsulation of lactoferrin in the nanocapsules leads to delivery of lactoferrin into cheese mass.

Example 13: Delivery of vitamin B12 to cheese

Aim:

To investigate if the encapsulation of vitamin B12 in the nanocapsules leads to delivery of vitamin B12 into cheese mass.

Material and methods:

The nanocapsules were prepared as described in example 3, except that 2 mg/ml vitamin B12 instead of lactoferrin was added. DLS as described in example 3 was performed.

For enrichment of cheese, the nanocapsules were added to 2.9-3.1% fat non-homogenized milk. Before adding the rennet, the milk was further enriched with 0.2 mg/mL calcium chloride and acidified by adding 5 mg/mL citric acid.

Then, the milk was equilibrated at 32 °C for 20 minutes and 0.2 U/mL chymosin enzyme was added. Incubation took place at 32 °C for 15 minutes. The curd was cut, and further incubation takes place at 32 °C for 15 min. During the incubation process, the curd was cut in 5 minutes intervals. The cheese mass
5 was then separated from whey by filtration.

Results:

DLS shows, that vitamin B12 can be incorporated in nanocapsules. The vitamin B12 nanocapsules are smaller compared to lactoferrin nanocapsules.
10 Furthermore, cheese fortified with vitamin B12 nanocapsules changes colour (Figure 18).

Conclusion:

The inventors of the present disclosure have shown that the
15 encapsulation of vitamin B12 in the nanocapsules leads to delivery of vitamin B12 into cheese mass.

Example 14: Stability of milk when fortified by nanocapsules

20 Aim:

To investigate the stability of milk when fortified by nanocapsules.

Material and methods:

The nanocapsules were prepared as described in example 3. For the
25 enrichment of milk, the nanocapsules at a concentration of 5 vol %, were added to the 3% fat milk. Gentle mixing via ten times inversion was performed for complete dispersion. Milk was stored at 4 °C for stability assay. The stability assay was performed by visual inspection, particle size analyser and SDS-PAGE. Controls were made with free lactoferrin, liposomes, buffer, or κ -casein.

30

Particle Size Analyzer: The particle size distribution was measured by using a LS230 laser diffraction particle size analyzer (Beckman Coulter, Inc., California, USA). A refractive index (RI) 1.46 for fat such as milk fat and olive

oil and 1.33 for water were used to calculate the droplet size. The droplet diameter profile of the samples was measured at least three times.

Results:

- 5 Milk comprising nanocapsules is stable against aggregation or proteolytic degradation for at least up to 12 weeks as compared with less than 2 weeks in the absence of nanocapsules (Figure 19).

Conclusion:

- 10 The inventors of the present disclosure have shown that nanocapsules can increase the stability of milk.

Example 15: Stability of apple juice and protein cargo when fortified by nanocapsules

- 15 Aim:

To investigate the stability of apple juice when fortified by nanocapsules.

Material and methods:

- 20 The nanocapsules were prepared as described in example 3. For the enrichment of apple juice, the nanocapsules at a concentration of 5 vol %, were added to the apple juice. Gentle mixing via ten times inversion was performed for complete dispersion. Apple juice was stored at 4°C for stability assay. The stability assay was performed by visual inspection, particle size analyser as described in example 14 and SDS-PAGE. Controls were made with free
25 lactoferrin or buffer.

Results:

- 30 Fortification with nanocapsules makes the juice cloudy and produce a sediment that can be resuspended upon shaking. SDS-PAGE were run on both the precipitate and the supernatant showing that the cargo protein, lactoferrin, remains intact inside the capsules even in the sediment (Figure 20).

Conclusion:

The inventors of the present disclosure have shown that lactoferrin remains inside the capsules even in the sediment of the apple juice, which can be resuspended by shaking, and the juice stability remains unchanged.

5 Example 16: Stability of oat milk when fortified with nanocapsules

Aim:

To investigate the stability of oat milk when fortified by nanocapsules.

Material and methods:

10 The nanocapsules were prepared as described in example 3. For the enrichment of oat milk, the nanocapsules at a concentration of 5 vol %, were added to the oat milk. Gentle mixing via ten times inversion was performed for complete dispersion. Milk was stored at 4°C for stability assay. The stability assay was performed by visual inspection, particle size analyser as described
15 in example 14 and SDS-PAGE. Controls were made with free lactoferrin or buffer.

Results and Conclusion:

 The inventors of the present disclosure have shown that the oat milk
20 stability remains unchanged when fortified with nanocapsules (Figure 21).

Example 17: Stability of soybean milk when fortified with nanocapsules

Aim:

 To investigate the stability of soybean milk when fortified by
25 nanocapsules.

Material and methods:

 The nanocapsules were prepared as described in example 3. For the enrichment of soybean milk, the nanocapsules at a concentration of 5 vol %, were added to the soybean milk. Gentle mixing via ten times inversion was performed for complete dispersion. Milk was stored at 4°C for stability assay. The stability assay was performed by visual inspection, particle size analyser
30

as described in example 14 and SDS-PAGE. Controls were made with free lactoferrin or buffer.

Results and Conclusion:

- 5 The inventors of the present disclosure have shown that the soybean milk stability remains unchanged when fortified with nanocapsules (Figure 22).

Example 18: Stability of yoghurt when fortified with nanocapsules

Aim:

- 10 To investigate the stability of yoghurt when fortified by nanocapsules.

Material and methods:

- 15 The nanocapsules were prepared as described in example 3. For the enrichment of yoghurt, the nanocapsules at a concentration of 5 vol %, were added to the yoghurt. Gentle mixing via ten times inversion was performed for complete dispersion. Yoghurt was stored at 4°C for stability assay. The stability assay was performed by visual inspection, particle size analyser as described in example 14 and SDS-PAGE. Controls were made with free lactoferrin or buffer.

20

Results and Conclusion:

The inventors of the present disclosure have shown that the yoghurt stability remains unchanged when fortified with nanocapsules (Figure 23).

- 25 Example 19: Comparison of plant-based emulsions with milk-based emulsions

Aim:

To investigate if κ -casein emulsification properties are suited for a wide range of food-based systems.

30

Material and methods:

3.5 vol% olive oil with 15 mg/mL lecithin was mixed with 35 mg/mL κ -casein from bovine milk. The aqueous solution for casein was a buffer

containing 20 mM imidazole, and 20 mM sodium chloride and pH 6.5. Emulsions were prepared by T25 digital ULTRA-TURRAX homogenizer (IKA-Werke GmbH & Co. KG, Staufen, Germany). Sample were homogenized 15000 rpm, 15 min, 25 °C.

5

The particle size distribution of the emulsions was measured by using a LS230 laser diffraction particle size analyzer (Beckman Coulter, Inc., California, USA). A refractive index (RI) 1.46 for fat such as milk fat and olive oil and 1.33 for water were used to calculate the droplet size. The droplet diameter profile
10 of the samples was measured at least three times.

Results:

κ-casein-based emulsions using bovine κ-casein purified from dairy milk
15 as emulsifier give similar droplet size distribution as milk emulsion (Figure 24).

Conclusion:

The inventors of the present disclosure have shown that κ-casein emulsification is suited for a wide range of beverages including milk mimics.

20

Example 20: Stability of emulsions

Aim:

To investigate the stability of emulsions.

25

Material and methods:

The emulsions were prepared as described in example 19. The droplet size distribution was measured as described in example 19.

30

Results and Conclusion:

The inventors of the present disclosure have shown that bovine κ-casein stabilized emulsions are stable upon storage at both 4 and 25 °C for at least up to 7 weeks and 1 week respectively (Figure 25).

Example 21: Stability of emulsions upon freeze dryingAim:

5 To investigate the stability of emulsions upon freeze drying.

Material and methods:

The emulsions were prepared as described in example 19. The droplet size distribution was measured as described in example 19.

10

Results and Conclusion:

The inventors of the present disclosure have shown that bovine κ -casein stabilized emulsions are stable upon freeze drying at least after 7 weeks of re-suspension and storage at 4 °C (Figure 26).

15

Example 22: Chymosin cleavage of κ -casein in emulsionAim:

To investigate if κ -casein in the emulsion can be cleaved by chymosin

20

Material and methods:

The emulsions were prepared as described in example 19. 0.2 mg/mL calcium chloride, 5 mg/mL citric acid and 0.2 U/mL enzyme were added. SDS-PAGE was performed.

25

Results and Conclusion:

The inventors of the present disclosure have shown that κ -casein in the emulsion surface can be cleaved by chymosin inducing a precipitate that mirrors the dairy cheese curd (Figure 27).

30

Example 23: Stability of milk emulsionsAim:

To investigate the stability of milk fortified with κ -casein-based emulsions.

Material and methods:

- 5 The emulsions were prepared as described in example 19. Controls were made with free lecithin in oil, buffer, or κ -casein. The κ -casein stabilized emulsion was added at a concentration of 5 vol %, to the milk. Gentle mixing via ten times inversion was performed for complete dispersion. Milk was stored at 4 °C for stability assay.
- 10 The droplet size distribution was measured as described in example 19. SDS-PAGE was performed.

Results and Conclusion:

- 15 The inventors of the present disclosure have shown that addition of emulsions to milk for fortification does not change milk stability upon storage 4 °C (Figure 28).

Sequence overview

5

SEQ ID NO: 1: Bovine κ -casein

MMKSFFLVVTILALTLPFLGAQEQNQEQPIRCEKDERFFSDKIAKYIPI
QYVLSRYPSYGLNYYQQKPVALINNQFLPYPPYAKPAAVRSPAQILQWQVL
SNTVPAKSCQAQPTTMARHPHLSFMAIPPKKNQDKTEIPTINTIASGEPT
10 STPTTEAVESTVATLEDSPEVIESPPEINTVQVTSTAV

SEQ ID NO: 2: Recombinant bovine κ -casein

QEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYGLNYYQQKPV
ALINNQFLPYPPYAKPAAVRSPAQILQWQVLSNTVPAKSCQAQPTTMARHH
15 PHLSFMAIPPKKNQDKTEIPTINTIASGEPTSTPTTEAVESTVATLEDSPEVIE
SPPEINTVQVTSTAV

SEQ ID NO: 3: Recombinant donkey κ -casein

EVQNQEQTCTRKNDERFFDLKTVKYIPVYYVLNSSPRNEPIYYQHRL
20 AVLINNQHMPYQYYARPAAVRPHVQIPQWQVLPNIYPSTVVRHPRPHPSFIA
IPPKKLQEKTVIPKINTIATVEPTPIPTPEPTVNNVIPDASSEFIIASTPETTTV
PVTSPVV

References

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Raouche, S., Dobenesque, M., Bot, A. et al. Casein micelles as a
vehicle for iron fortification of foods. Eur Food Res Technol 229, 929–935
(2009). <https://doi.org/10.1007/s00217-009-1130-0>

30 Items

The invention may further be defined by the following items:

1. A dispersion, said dispersion comprising:

- 35 a) casein, wherein at least 60% of the casein is κ -casein; and
 b) phospholipids.

2. The dispersion according to item 1, wherein the dispersion is a nanocapsule comprising:
 - a) a shell comprising phospholipid and casein, wherein at least 60% of the casein is κ -casein; and
 - 5 b) a hydrophilic core.
3. A nanocapsule comprising:
 - a) a shell comprising phospholipid and casein, wherein at least 60% of the casein is κ -casein; and
 - 10 b) a hydrophilic core.
4. The dispersion according to item 1, wherein the delivering composition is an emulsion comprising oil in water droplets comprising:
 - a) a shell comprising casein, wherein at least 60% of the casein is κ -casein; and
 - 15 b) a hydrophobic core.
5. An emulsion comprising oil in water droplets comprising:
 - a) a shell comprising casein, wherein at least 60% of the casein is κ -casein; and
 - 20 b) a hydrophobic core.
6. The emulsion according to item 5, wherein the shell further comprises phospholipids.
- 25 7. The dispersion according to any one of the preceding items, wherein the κ -casein is purified from milk, such as from bovine milk, goat milk, sheep milk, camel milk, donkey milk, horse milk, reindeer milk, yak milk or human milk.
- 30 8. The dispersion according to any one of the preceding items, wherein the κ -casein is recombinant κ -casein.

9. The dispersion according to any one of the preceding items, wherein the κ -casein is recombinant κ -casein produced by a host cell or host organism comprising a heterologous nucleic acid encoding κ -casein.
- 5 10. The dispersion according to item 9, wherein the host cell or host organism is yeast, bacteria, fungi, algae, plants, insect or mammalian.
11. The dispersion according to item 9, wherein said host cell is a bacterial cell, such as *E. coli* and *B. subtilis*.
- 10 12. The dispersion according to any one of the preceding items, wherein the host organism is an alga, such as *C. reinhardtii* and *C. vulgaris*.
13. The dispersion according to item 9, wherein said host cell is a yeast cell,
15 such as a yeast cell of the genus *Saccharomyces* or *Pichia*.
14. The dispersion according to any one of the preceding items, wherein the κ -casein is a κ -casein of SEQ ID NO: 1 or a functional homologue of said κ -casein sharing at least 70%, such as at least 80%, for example at least
20 90%, such as at least 95% sequence identity therewith.
15. The dispersion according to any one of the items 1 to 5, wherein the κ -casein is a κ -casein of SEQ ID NO: 2 or a functional homologue of said κ -casein sharing at least 70%, such as at least 80%, for example at least
25 90%, such as at least 95% sequence identity therewith.
16. The dispersion according to any one of the items 1 to 5, wherein the κ -casein is a κ -casein of SEQ ID NO: 3 or a functional homologue of said κ -casein sharing at least 70%, such as at least 80%, for example at least
30 90%, such as at least 95% sequence identity therewith.
17. The dispersion according to any one of the preceding items, wherein at least 70%, such as at least 80%, such as at least 90% of the caseins are κ -caseins.

18. The dispersion according to any one of the preceding items, wherein at most 40%, such as at most 30%, such as at most 20%, such as at most 10%, such as at most 5%, such as at most 1%, such as at most 0,1 % of the caseins are α S1-casein.
- 5 19. The dispersion according to any one of the preceding items, wherein at most 40%, such as at most 30%, such as at most 20%, such as at most 10%, such as at most 5%, such as at most 1%, such as at most 0,1 % of the caseins are α S2-casein.
- 10 20. The dispersion according to any one of the preceding items, wherein at most 40%, such as at most 30%, such as at most 20%, such as at most 10%, such as at most 5%, such as at most 1%, such as at most 0,1 % of the caseins are β -casein.
- 15 21. The dispersion according to any one of the preceding items, wherein at most 40%, such as at most 30%, such as at most 20%, such as at most 10%, such as at most 5%, such as at most 1%, such as at most 0,1 % of the caseins are α S1-casein, α S2-casein and β -casein.
- 20 22. The dispersion according to any one of the preceding items, wherein the casein does not comprise α S1-casein, α S2-casein and/or β -casein.
23. The dispersion according to any one of the preceding items, wherein the
- 25 casein does not comprise any of α S1-casein, α S2-casein and β -casein.
24. The dispersion according to any one of the preceding items, wherein the phospholipids are one or more phospholipids selected from the group consisting of synthetic phospholipids, animal-based phospholipids and
- 30 plant-based phospholipids.
25. The dispersion according to any one of the preceding items, wherein the phospholipids are a mixture of glycerophospholipids, for example

phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, or phosphatidic acid.

- 5 26. The dispersion according to any one of the preceding items, wherein the phospholipids are mixed with oil.
27. The dispersion according to any one of the preceding items, wherein the phospholipids comprise or consist of lecithin, for example dihexanoyl-L-
- 10 alpha-lecithin, dioctanoyl-L-alpha-lecithin, didecanoyl-L-alpha-lecithin, didodecanoyl-L-alpha-lecithin, ditetradecanoyl-L-alpha-lecithin, dihexadecanoyl-L-alpha-lecithin, dioctadecanoyl-L-alpha-lecithin, dioleoyl-L-alpha-lecithin, dilinoleoyl-L-alpha-lecithin, or alpha-palmitol.
- 15 28. The dispersion according to any one of the preceding items, wherein the phospholipids or the lecithin are derived from one or more source selected from the group consisting of egg yolk, marine foods, soybeans, milk, rapeseed, cottonseed, and sunflower oil.
- 20 29. The dispersion according to any one of the preceding items, wherein the phospholipid is a lecithin, which is pure or food grade lecithin.
30. The dispersion according to any one of the preceding items, wherein the phospholipids are acetone-insoluble phosphatides.
- 25 31. The dispersion according to any one of the preceding items, wherein the dispersion comprises an added value compound.
32. The dispersion according to any one of items 2 to 3 and 7 to 31, wherein
- 30 the hydrophilic core comprises a water-soluble added value compound.
33. The dispersion according to any one of the items 4 to 31, wherein the hydrophobic core comprises an oil-soluble added value compound.

34. The dispersion according to any one of the preceding items, wherein at least one added value compound is a food supplement.
35. The dispersion according to any one of the preceding items, wherein at least one added value compound is a vitamin, such as vitamin A, vitamin B, vitamin C, vitamin D, vitamin E or vitamin K.
36. The dispersion according to according to any one of the preceding items, wherein at least one added value compound is an antioxidant, such as vitamin A, vitamin C, vitamin E, xanthophylls, such as zeaxanthin or lutein, polyphenols or flavonoids such as resveratrol or quercetin.
37. The dispersion according to any one of the preceding items, wherein at least one added value compound is a protein, such as an iron carrying protein, such as hemoglobin or lactoferrin.
38. The dispersion according to according to any one of the preceding items, wherein at least one added-value compound is an enzyme, such as amylase or β -galactosidase.
39. The dispersion according to according to any one of the preceding items, wherein at least one added value compound is a fatty acid, such as omega 3 fatty acid or omega 6 fatty acid.
40. The dispersion according to any one of the preceding items, wherein at least one added value compound is a therapeutically active substance, such as antibiotics, peptides and/or hormones.
41. The dispersion according to any one of the items 1 to 40, wherein the dispersion when mixed with food products and/or beverages increases the stability of said food products and/or beverages.
42. The dispersion according to any one of the items 1 to 41, wherein the dispersion when mixed with milk increases the stability of said milk.

43. A method of producing the nanocapsules according to any one of items 2 to 3 and 7 to 42, wherein the method comprises the following steps:
- a) preparing a lipid film, wherein the lipid film preferably comprises phospholipids;
 - 5 b) preparing a hydrophilic solution comprising casein, wherein at least 60% of the casein is κ -casein and optionally an added value compound;
 - c) solubilizing said lipid film prepared in step a) in the solution prepared in step b); and
 - 10 d) obtaining a solution containing the nanocapsules.
44. The method according to item 43, wherein the lipid film of step a) is prepared by dissolving phospholipids, for example lecithin in an organic solvent, and subsequently removing said organic solvent, for example
- 15 evaporating the organic solvent.
45. The method according to item 44, wherein the organic solvent in step a) is chloroform or hexane.
- 20 46. The method according to any one of items 43 to 45, wherein the lipid film in step a) is prepared by dissolving lecithin.
47. The method according to any one of items 43 to 46, wherein the hydrophilic solution of step b) comprises an added value compound.
- 25 48. The method according to any one of items 43 to 47, wherein the solution in step b) is prepared by resuspending an added value compound in a buffer and mixing said buffer with a solution comprising said caseins.
- 30 49. The method according to any one of items 43 to 48, wherein the hydrophilic solution of step b) is prepared by a method comprising resuspending κ -casein in an aqueous buffer.

50. The method according to item 49, wherein the concentration of κ -casein in the aqueous solution of step b) is at least 0.5 mg/ml, such as at least 1 mg/ml, such as at least 1.5 mg/ml, such as at least 2 mg/ml, such as at least 2.5 mg/ml, such as at least 3 mg/ml, such as at least 4 mg/ml, such as at least 5 mg/ml, such as at least 10 mg/ml.
51. The method according to item 49, wherein the concentration of κ -casein in the aqueous solution of step b) is at most 20 mg/ml, such as at most 15 mg/ml, such as at most 10 mg/ml, such as at most 5 mg/ml, such as at most 3 mg/ml, such as at most 2.5 mg/ml, such as at most 2 mg/ml, such as at most 1.5 mg/ml, such as at most 1 mg/ml, such as at most 0.5 mg/ml.
52. The method according to item 49, wherein the concentration of κ -casein in the aqueous solution of step b) is between 1 mg/ml and 10 mg/ml.
53. The method according to any one of items 43 to 52, wherein in step d) of the method the solution comprises phospholipids at a concentration of at least 0.1 mg/ml, such as at least 0.5 mg/ml, such as at least 1 mg/ml, such as at least 2 mg/ml, such as at least 5 mg/ml, such as at least 8 mg/ml, such as at least 10 mg/ml, such as at least 10 mg/ml of the total solution.
54. The method according to any one of items 43 to 52, wherein in step d) of the method the solution comprises phospholipids at a concentration of at most 20 mg/ml, such as at most 10 mg/ml, such as at most 8 mg/ml, such as at most 5 mg/ml, such as at most 2 mg/ml, such as at most 1 mg/ml, such as at most 0.5 mg/ml, such as at most 0.1 mg/ml of the total solution.
55. The method according to any one of items 43 to 54, wherein in step c) the lipid film is solubilized by mixing the lipid film of step a) with the solution of step b), wherein the mixing is performed by one or more of the methods of a group comprising vortexing, homogenization, bath sonication and tip sonication.

56. The method according to any one of items 43 to 55, wherein in step d) a solution containing nanocapsules comprising a shell made of phospholipid and κ -casein and a core comprising at least one water-soluble added value compound is obtained.
57. The method according to any one of items 43 to 56, wherein in step d) the nanocapsules are eluted in a buffer.
58. A method of producing the emulsion according to any one of items 4 to 42, wherein the method comprises the following steps:
- a) providing a hydrophobic solution, preferably comprising an oil;
 - b) preparing a hydrophilic solution comprising casein, wherein at least 60% of the casein is κ -casein;
 - c) Emulsifying said hydrophobic solution with said casein solution;
 - d) thereby obtaining an emulsion.
59. The method according item 58, wherein the hydrophobic solution in step a) comprises oil and a phospholipid, such as lecithin.
60. The method according to item 59, wherein the oil is olive oil, rapeseed oil, soybean oil, sunflower oil or corn oil.
61. The method according to any one of items 59 to 60, wherein the concentration of the oil is at least 0.5 vol%, such as at least 1 vol%, such as at least 5 vol%, such as at least 10 vol%, such as at least 15 vol%, such as at least 20 vol% of the hydrophobic solution.
62. The method according to any one of items 59 to 60, wherein the concentration of the oil is at most 20 vol%, such as at most 15 vol%, such as at most 10 vol%, such as most 5 vol% of the hydrophobic solution.

63. The method according to any one of items 59 to 60, wherein the concentration of the oil is between 0.5 vol% and 10 vol% of the hydrophobic solution.
- 5 64. The method according to any one of items 58 to 63, wherein the hydrophobic solution of step a) comprises an added value compound.
65. The method according to any one of items 58 to 64, wherein the concentration of κ -casein in the solution of step b) is at least 10 mg/ml,
10 such as at least 15 mg/ml, such as at least 20 mg/ml, such as at least 25 mg/ml, such as at least 30 mg/ml, such as at least 35 mg/ml.
66. The method according to any one of items 58 to 64, wherein the concentration of κ -casein in the solution of step b) is at most 40 mg/ml,
15 such as at most 35 mg/ml, such as at most 30 mg/ml, such as at most 25 mg/ml, such as at most 20 mg/ml, such as at most 15 mg/ml, such as at most 10 mg/ml.
67. The method according to any one of items 58 to 64, wherein the
20 concentration of κ -casein in the solution of step b) is between 20 mg/ml and 40 mg/ml.
68. The method according to any one of items 58 to 67, wherein in step d) an emulsion is obtained.
- 25 69. The method according to any one of the items 43 to 68, wherein the phospholipid is as defined in any one of items 24 to 30.
70. The method according to any one of the items 43 to 69, wherein the
30 added value compound is as defined in any one of items 24 to 30.
71. The method according to any one of items 43 to 70, wherein the hydrophilic solution of step b) or to the hydrophobic solution of step a) comprises the added value compound at a concentration of at least 0.1
35 mg/ml, such as at least 0.5 mg/ml, such as at least 1 mg/ml, such as at

least 1.5 mg/ml, such as at least 2 mg/ml, such as at least 3 mg/ml, such as at least 4 mg/ml, such as at least 5 mg/ml.

72. The method according to any one of items 43 to 71, wherein the
5 hydrophilic solution of step b) or to the hydrophobic solution of step a) comprises the added value compound at a concentration of at most 20 mg/ml, such as at most 10 mg/ml, such as at most 5 mg/ml, such as at most 4 mg/ml, such as at most 3 mg/ml, such as at most 2 mg/ml, such as at most 1 mg/ml, such as at most 0.5 mg/ml total protein.
- 10 73. The method according to item 43 to 72, wherein the hydrophilic solution of step b) or to the hydrophobic solution of step a) comprises the added value compound at a concentration between 0.1 mg/ml and 10 mg/ml.
- 15 74. The method according to any one of items 43 to 73, wherein κ -casein is as defined in any one of items 7 to 23.
75. The method according to any one of the items 43 to 74, wherein the solution in step b) comprises a buffer.
- 20 76. A nanocapsule prepared by the method according to any one of the items 43 to 57 and 74 to 75.
77. An emulsion prepared by the method according to any one of the items 58
25 to 75.
78. Use of the dispersion, the nanocapsules or the emulsion according to any one of the items 1 to 42 for the transport and/or encapsulation of added value compound(s).
- 30 79. Use of the dispersion, the nanocapsules or the emulsion according to any one of the items 1 to 42 for enrichment of a food product and/or beverages with an added value compound.

80. The use according to item 79, wherein the food product and/or beverages are dairy products or plant-based dairy alternative analogues or mixtures thereof.
- 5 81. The use according to item 79, wherein the food product and/or beverages are milk, milk analogues, yoghurt, yoghurt analogues, cheese, cheese analogues, juice or mixtures of any of the aforementioned.
82. Use of the dispersion, the nanocapsules or the emulsion according to any
10 one of the items 1 to 42 for increasing the nutritional value in food products, such as cheese, cheese analogues and/or beverages.
83. A method of producing a food product and/or beverages, wherein the method comprises the following steps:
- 15 a) Providing a food product, a beverage, food product ingredient or a beverage base;
- b) contacting said food product, beverage, food product ingredient or beverage base with the dispersion, the nanocapsules or the emulsion according to any one of the items 1 to 42,
- 20 c) processing the mixture of step b) into a product or a beverage.
84. The method according to item 83, wherein the food product or the beverage is milk, milk analogue, yoghurt, yoghurt analogue or mixtures of any of the aforementioned.
- 25 85. The method according to item 83, wherein the food product is cheese, a cheese analogue or mixtures thereof.
86. The method according to item 83, wherein the dispersion, the nanocapsule
30 and/or the emulsion are added to said in step b) to the food product, beverage, food product ingredient or beverage base at a concentration of at least 3 vol%, such as at least 4 vol%, such as at least 5 vol% of the food product, beverage, food product ingredient or beverage base .

87. The method according to item 83, wherein the dispersion, the nanocapsule and/or the emulsion are added in step b) to the food product, beverage, food product ingredient or beverage base at a concentration of at most 50 vol%, such as at most 40 vol%, such as at most 30 vol%, such as at most 10 vol%, such as at most 8 vol%, such as at most 6 vol%, such as at most 5 vol% of the food product, beverage, food product ingredient or beverage base.
88. The method according to item 83, wherein the dispersion, the nanocapsule and/or the emulsion are added in step b) to the food product, beverage, food product ingredient or beverage base at a concentration of 5 vol% of the food product, beverage, food product ingredient or beverage base.
89. A method of producing cheese, wherein the method comprises the following steps:
- a) Providing milk or milk analogue comprising the dispersion, the nanocapsules or the emulsion according to any one of the items 1 to 42;
 - b) processing said milk into cheese or cheese analogue.
90. The method according to item 89, wherein the provided milk in the method of producing cheese in step a) is non-homogenized or homogenized milk.
91. The method according to any one of items 89 to 90, wherein step b) comprises the steps of
- Adding calcium chloride to the milk or milk analogue of step a);
 - Acidifying the milk or milk analogue;
 - Equilibrating the milk or milk analogue;
 - Adding chymosin to the milk or milk analogue;
 - Incubating the milk or milk analogue to obtain curd;
 - Incubating the curd to obtain cheese or cheese analogue; and

- Separating the cheese mass or cheese analogue mass from whey.

92. The method according to any one of items 89 to 91, wherein the cheese
5 mass or cheese analogue mass and the whey are separated in step i) by
filtration.

CLAIMS

1. A dispersion, said dispersion comprising:
 - a) casein, wherein at least 60% of the casein is κ -casein; and
 - 5 b) phospholipids.
2. A nanocapsule comprising:
 - a) a shell comprising phospholipid and casein, wherein at least 60% of the casein is κ -casein; and
 - 10 b) a hydrophilic core.
3. An emulsion comprising oil in water droplets comprising:
 - a) a shell comprising casein, wherein at least 60% of the casein is κ -casein; and
 - 15 b) a hydrophobic core.
4. The emulsion according to claim 3, wherein the shell further comprises phospholipids.
- 20 5. The dispersion according to any one of the preceding claims, wherein the κ -casein is purified from milk, such as from bovine milk, goat milk, sheep milk, camel milk, donkey milk, horse milk, reindeer milk, yak milk or human milk or wherein the κ -casein is recombinant κ -casein.
- 25 6. The dispersion according to any one of the preceding claims, wherein the κ -casein is a κ -casein
 - a) of SEQ ID NO: 1 or a functional homologue of said κ -casein sharing at least 70%, such as at least 80%, for example at least 90%, such as at least 95% sequence identity therewith; or
 - 30 b) a κ -casein of SEQ ID NO: 2 or a functional homologue of said κ -casein sharing at least 70%, such as at least 80%, for example

at least 90%, such as at least 95% sequence identity therewith;
or

- 5 c) a κ -casein of SEQ ID NO: 3 or a functional homologue of said κ -casein sharing at least 70%, such as at least 80%, for example at least 90%, such as at least 95% sequence identity therewith.

7. The dispersion according to any one of the preceding claims, wherein at least 70%, such as at least 80%, such as at least 90% of the caseins are κ -caseins.

10

8. The dispersion according to any one of the preceding claims, wherein at most 40%, such as at most 30%, such as at most 20%, such as at most 10%, such as at most 5%, such as at most 1%, such as at most 0,1 % of the caseins are α S1-casein, α S2-casein and β -casein.

15

9. The dispersion according to any one of the preceding claims, wherein the phospholipids comprise or consist of lecithin, for example dihexanoyl-L-alpha-lecithin, dioctanoyl-L-alpha-lecithin, didecanoyl-L-alpha-lecithin, didodecanoyl-L-alpha-lecithin, ditetradecanoyl-L-alpha-lecithin, dihexadecanoyl-L-alpha-lecithin, dioctadecanoyl-L-alpha-lecithin, dioleoyl-L-alpha-lecithin, dilinoleoyl-L-alpha-lecithin, or alpha-palmitol.

20

10. The dispersion according to any one of claims 2 and 4 to 9, wherein the hydrophilic core comprises a water-soluble added value compound.

25

11. The dispersion according to any one of the claims 3 to 9, wherein the hydrophobic core comprises an oil-soluble added value compound.

12. The dispersion according to any one of the preceding claims, wherein at least one added value compound is a food supplement and/or a therapeutically active substance.

30

13. The dispersion according to any one of the preceding claims, wherein the dispersion when mixed with food products and/or beverages increases the stability of said food products and/or beverages.
- 5 14. A method of producing the nanocapsules according to any one of claims 2 and 5 to 13, wherein the method comprises the following steps:
- a) preparing a lipid film;
 - b) preparing a hydrophilic solution comprising casein, wherein at least 60% of the casein is κ -casein;
 - 10 c) solubilizing said lipid film prepared in step a) in the solution prepared in step b); and
 - d) obtaining a solution containing the nanocapsules.
- 15 15. The method according to claim 14, wherein the lipid film comprises phospholipids.
16. A method of producing the emulsion according to any one of claims 3 to 13, wherein the method comprises the following steps:
- a) providing a hydrophobic solution, preferably comprising an oil;
 - 20 b) preparing a hydrophilic solution comprising casein, wherein at least 60% of the casein is κ -casein;
 - c) Emulsifying said hydrophobic solution with said casein solution;
 - d) thereby obtaining an emulsion.
- 25 17. The method according claim 16, wherein the hydrophobic solution in step a) comprises oil and a phospholipid, such as lecithin.
- 30 18. The method according to any one of claims 14 to 17, wherein the hydrophilic solution of step b) in the method of producing nanocapsules or the hydrophobic solution of step a) in the method of producing emulsions comprise an added value compound.

19. Use of the dispersion, the nanocapsules or the emulsion according to any one of the claims 1 to 13 for the transport and/or encapsulation of added value compound(s).
- 5 20. Use of the dispersion, the nanocapsules or the emulsion according to any one of the claims 1 to 13 for enrichment of a food product and/or beverages with an added value compound.
21. The use according to claim 20, wherein the food product and/or beverages
10 are dairy products or plant-based dairy alternative analogues or mixtures thereof.
22. A method of producing a food product and/or beverages, wherein the method comprises the following steps:
- 15 a) Providing a food product, a beverage, food product ingredient or a beverage base;
b) contacting said food product, beverage, food product ingredient or beverage base with the dispersion, the nanocapsules or the emulsion according to any one of the claims 1 to 13,
20 c) processing the mixture of step b) into a product or a beverage.
23. A method of producing cheese or cheese analogue, wherein the method comprises the following steps:
- 25 a) Providing milk or milk analogue comprising the dispersion, the nanocapsules or the emulsion according to any one of the claims 1 to 13;
b) processing said milk or milk analogue into cheese or cheese analogue.

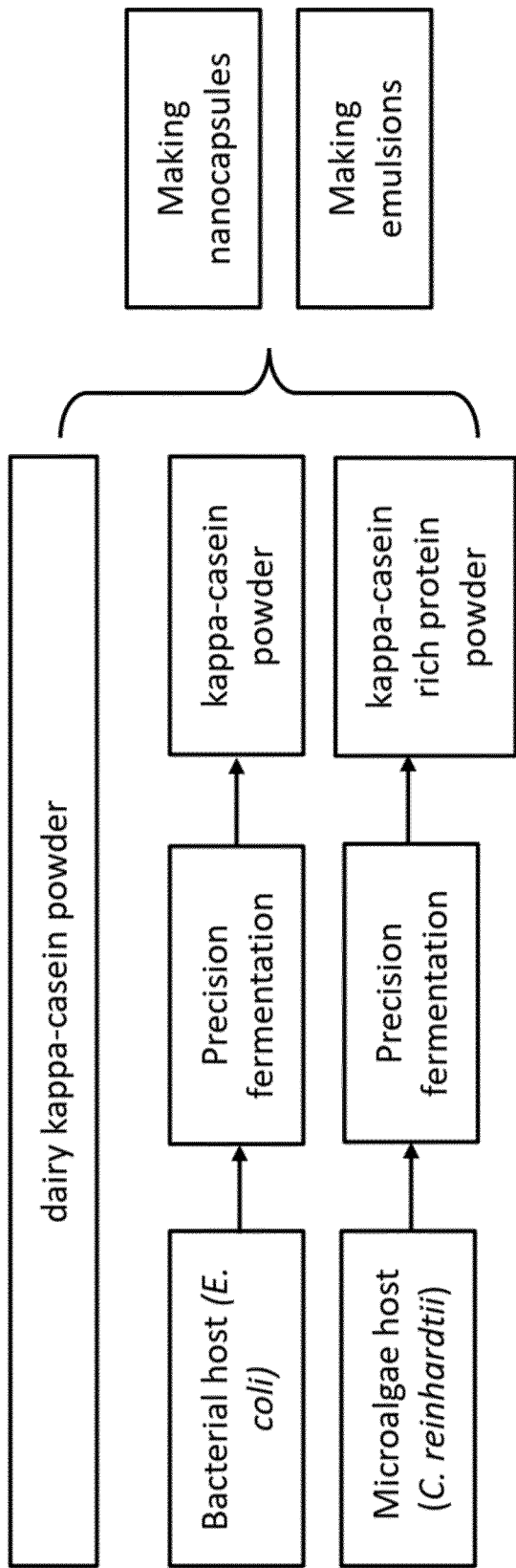
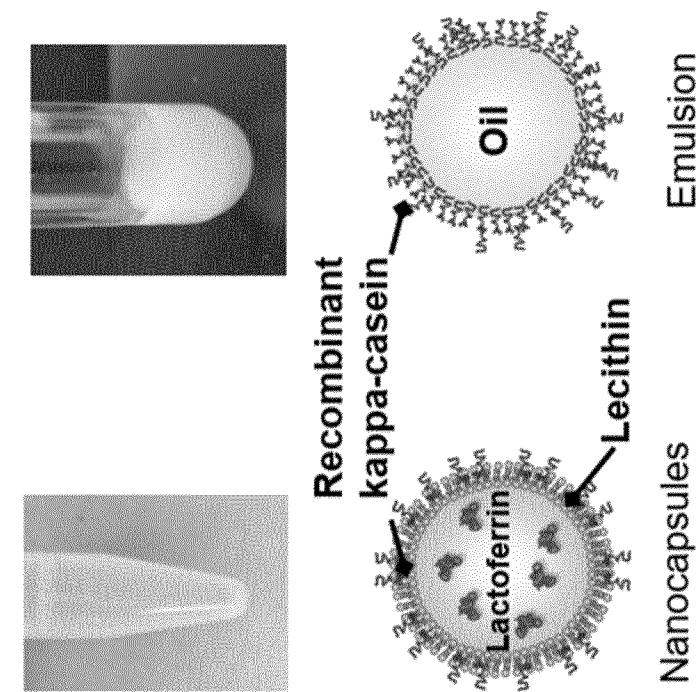
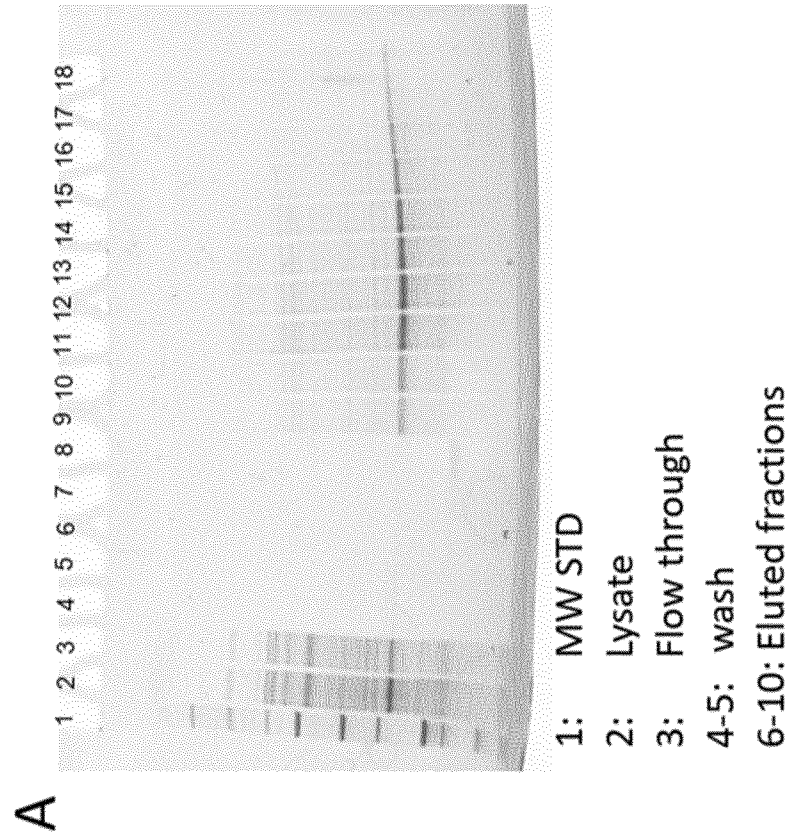


Fig. 1



B



A

Fig. 3

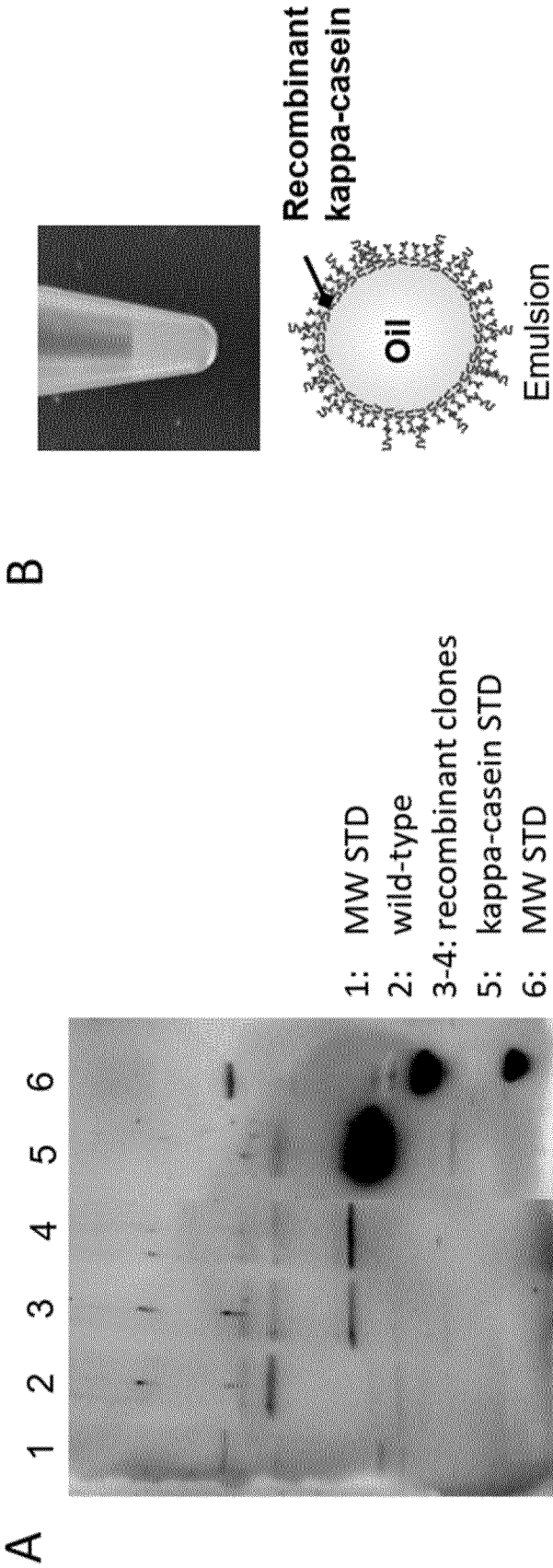
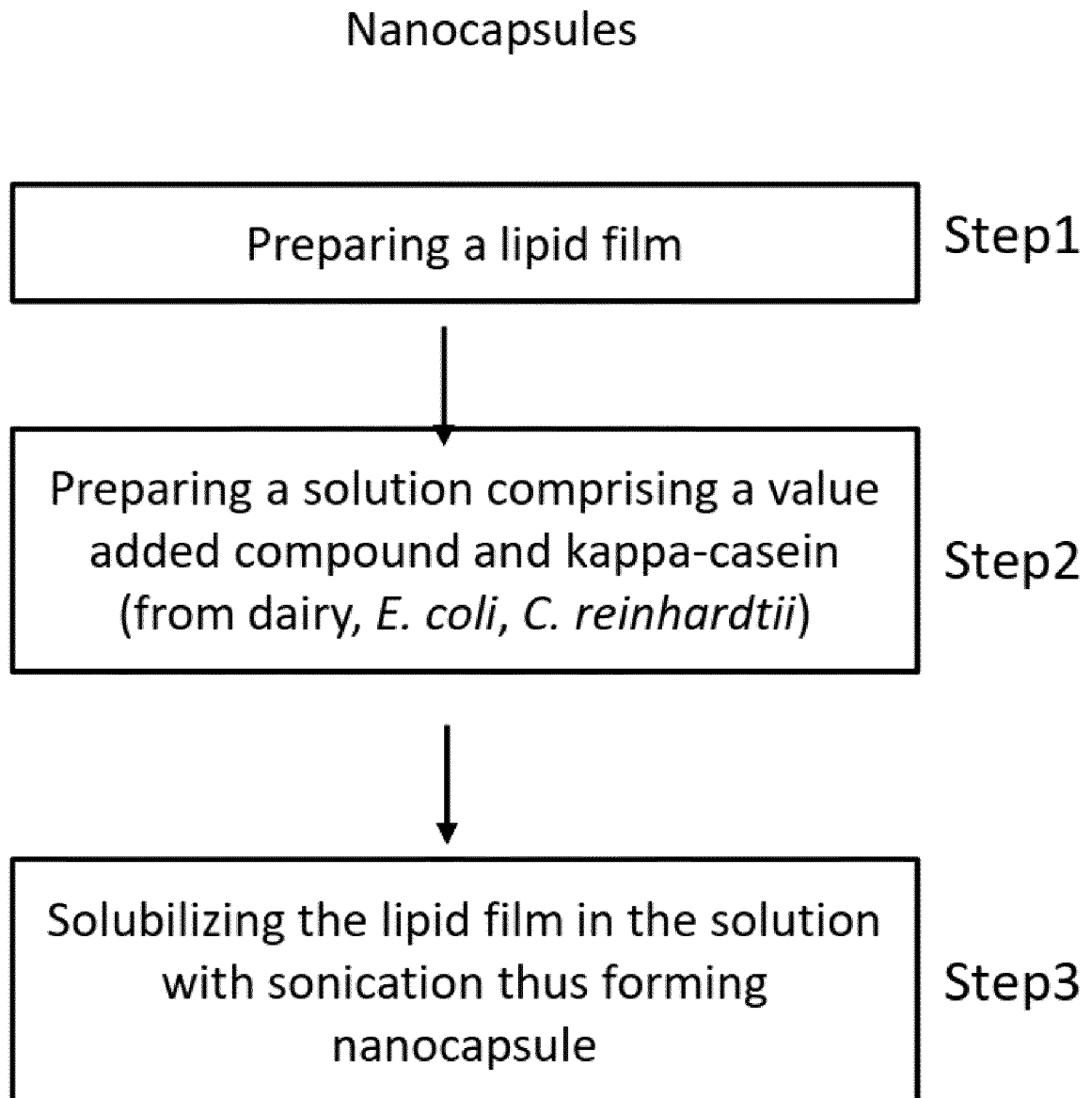


Fig. 4



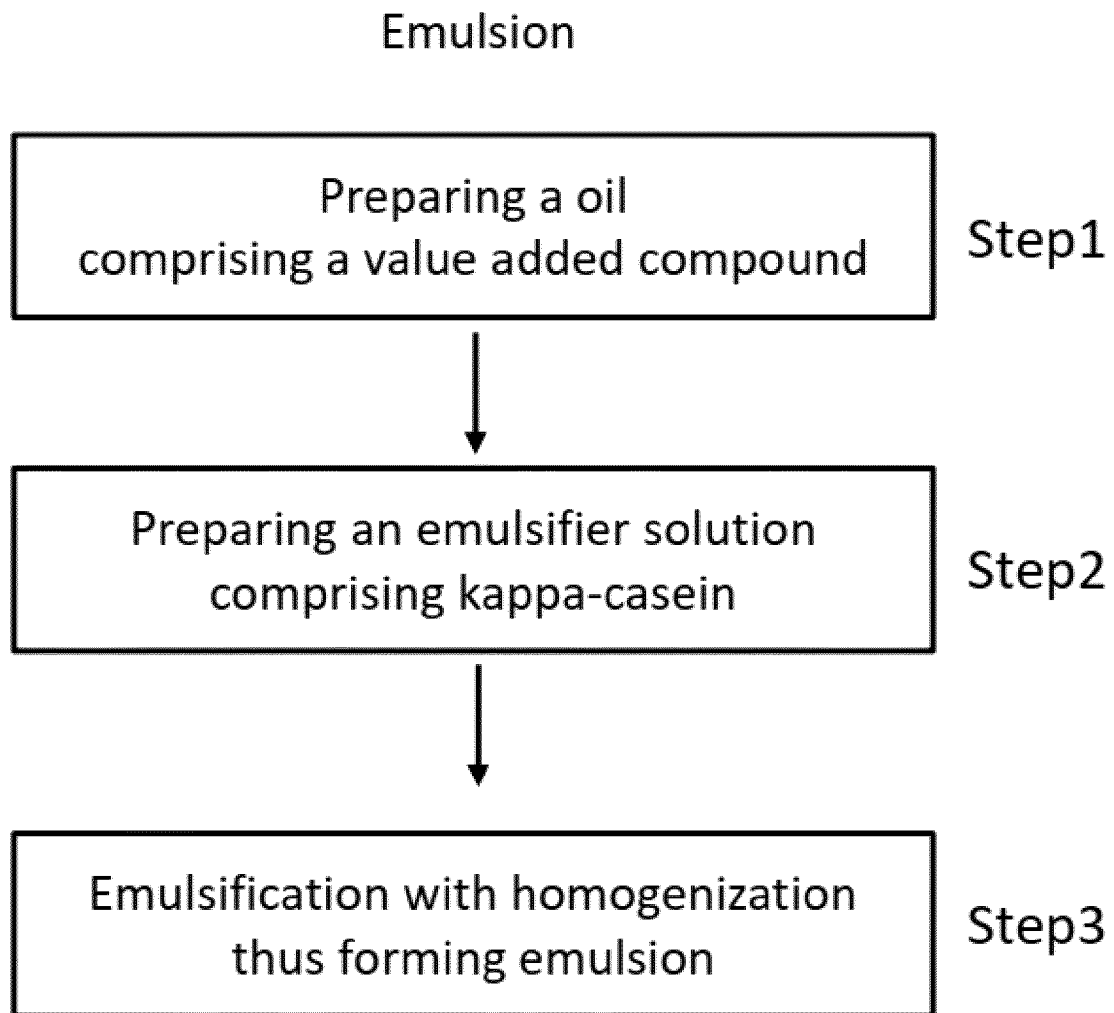


Fig. 6

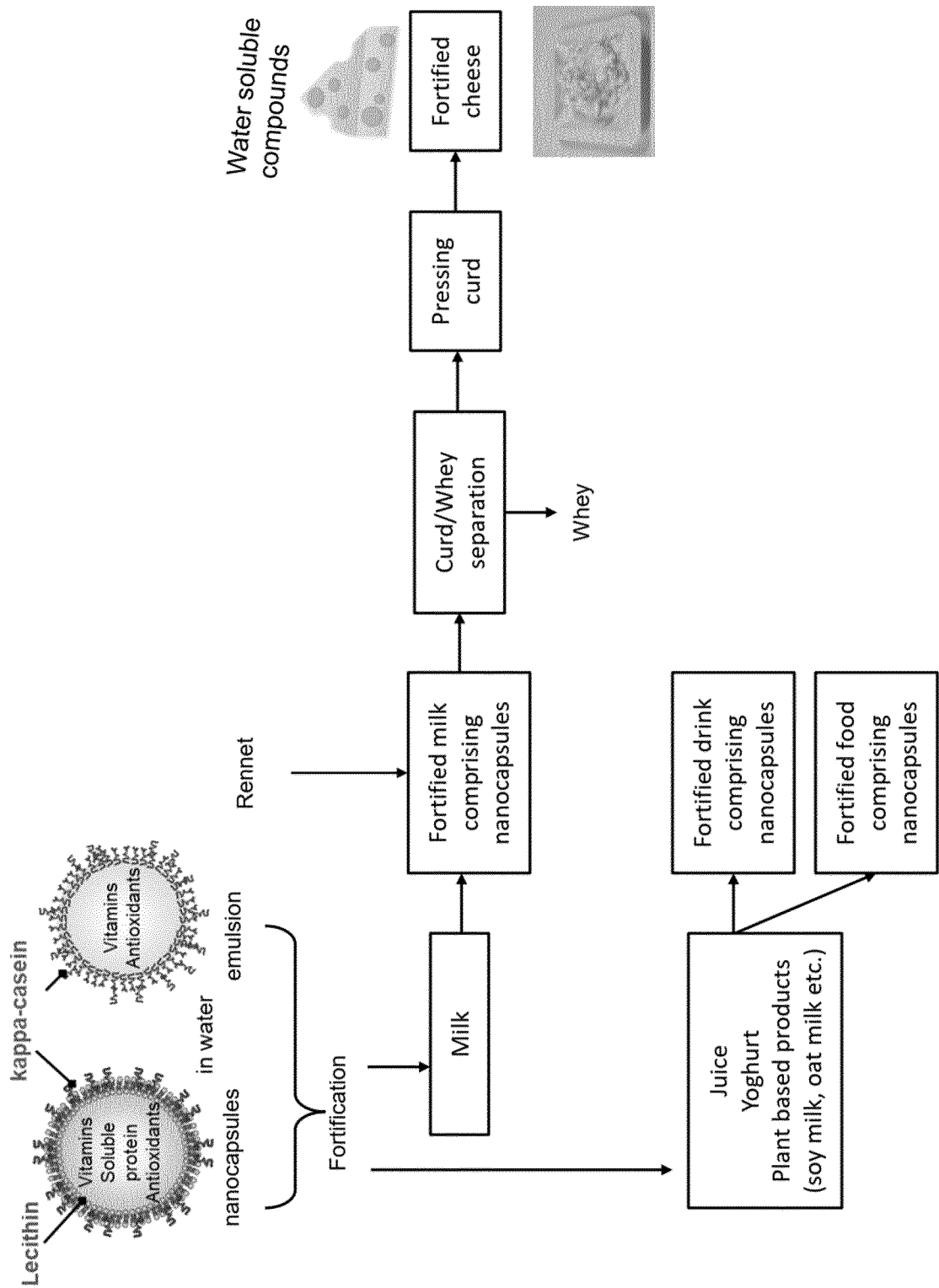


Fig. 7

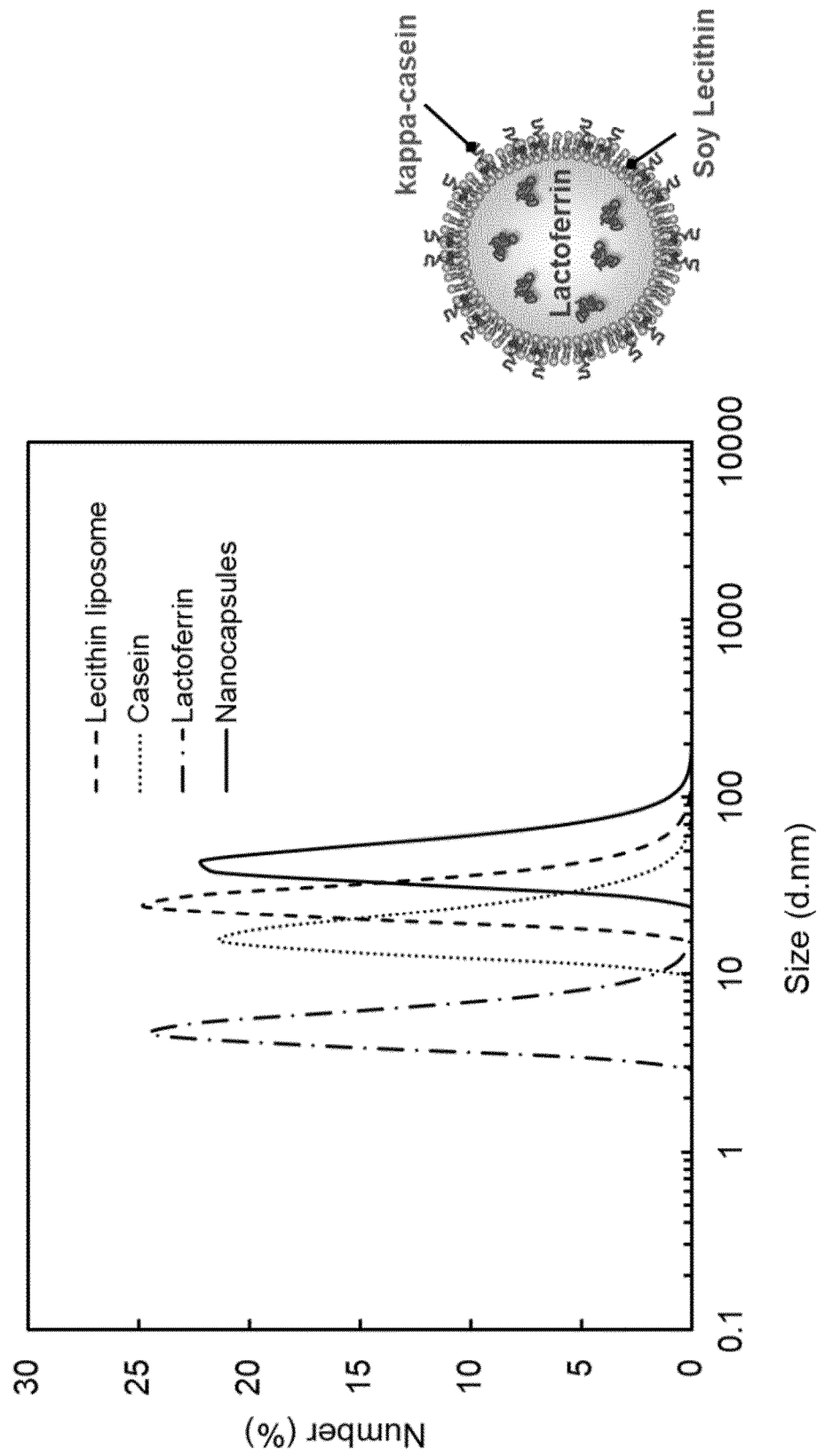


Fig.8

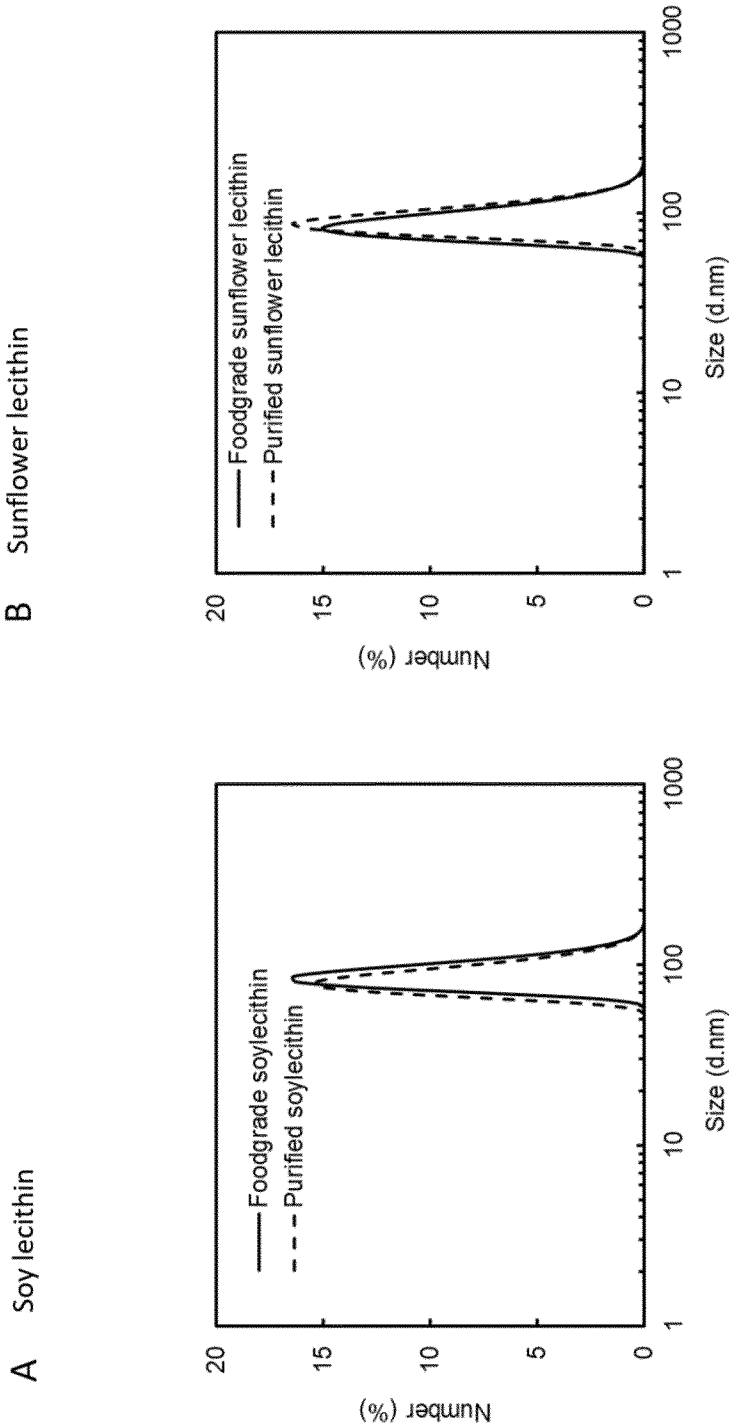


Fig. 9

A pH B NaCl

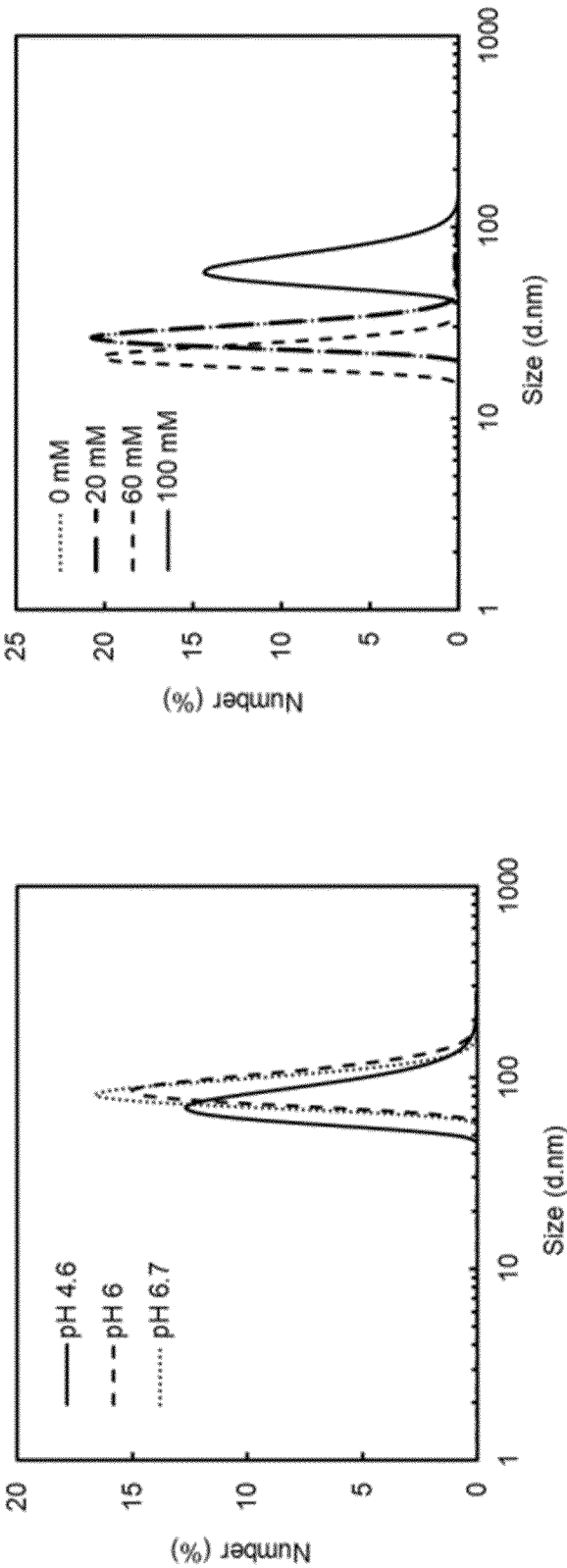


Fig. 10

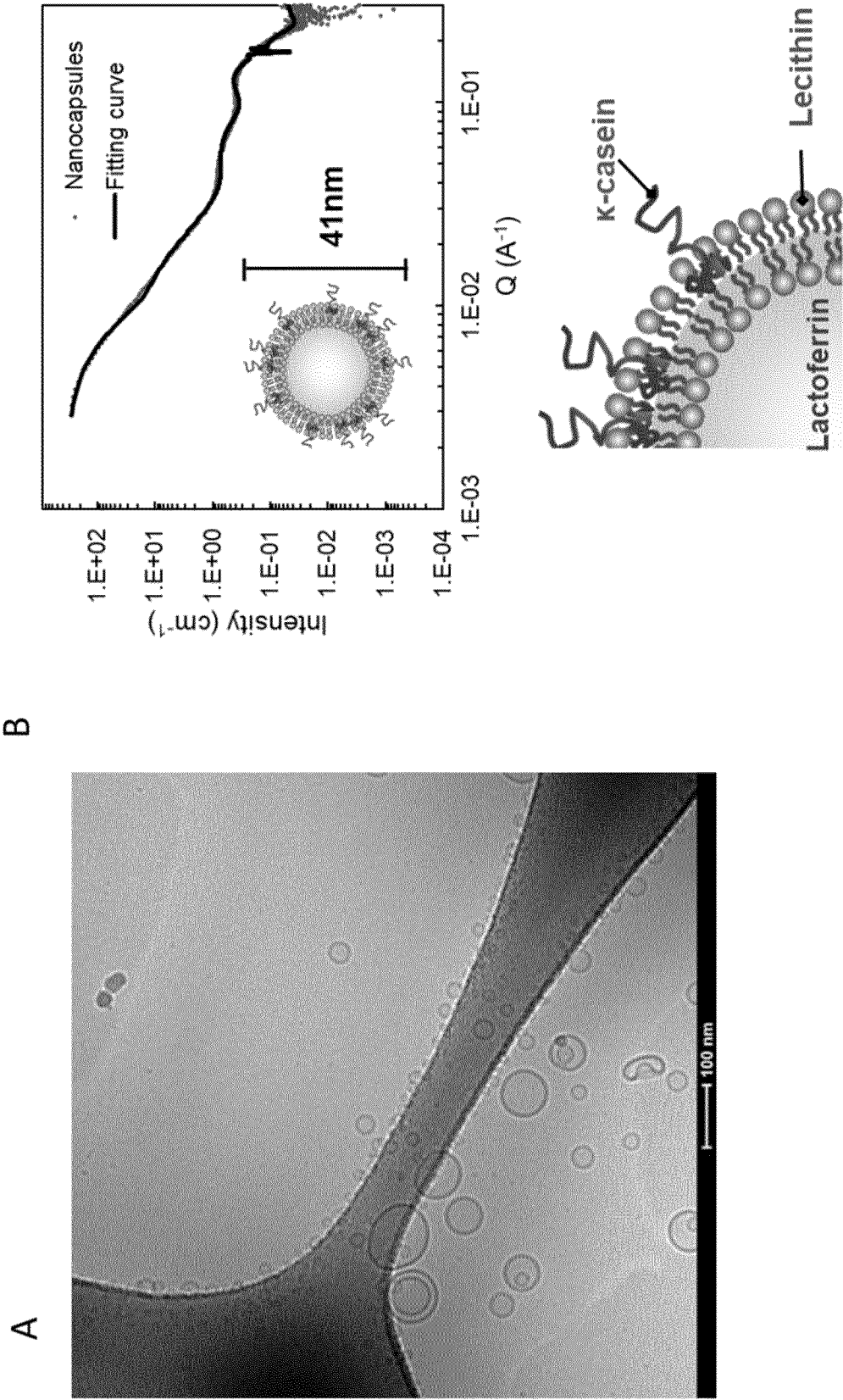


Fig. 11

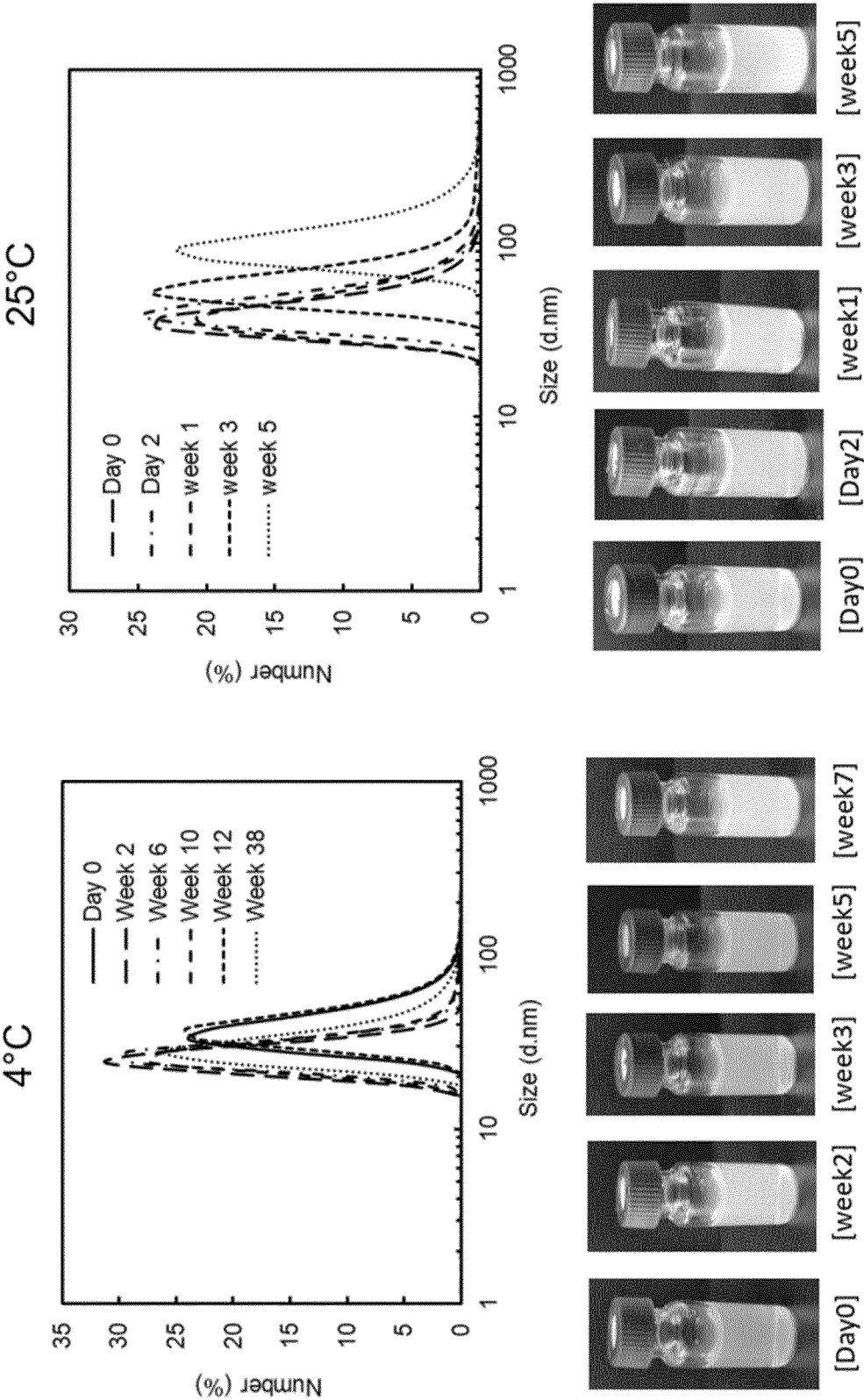


Fig. 12

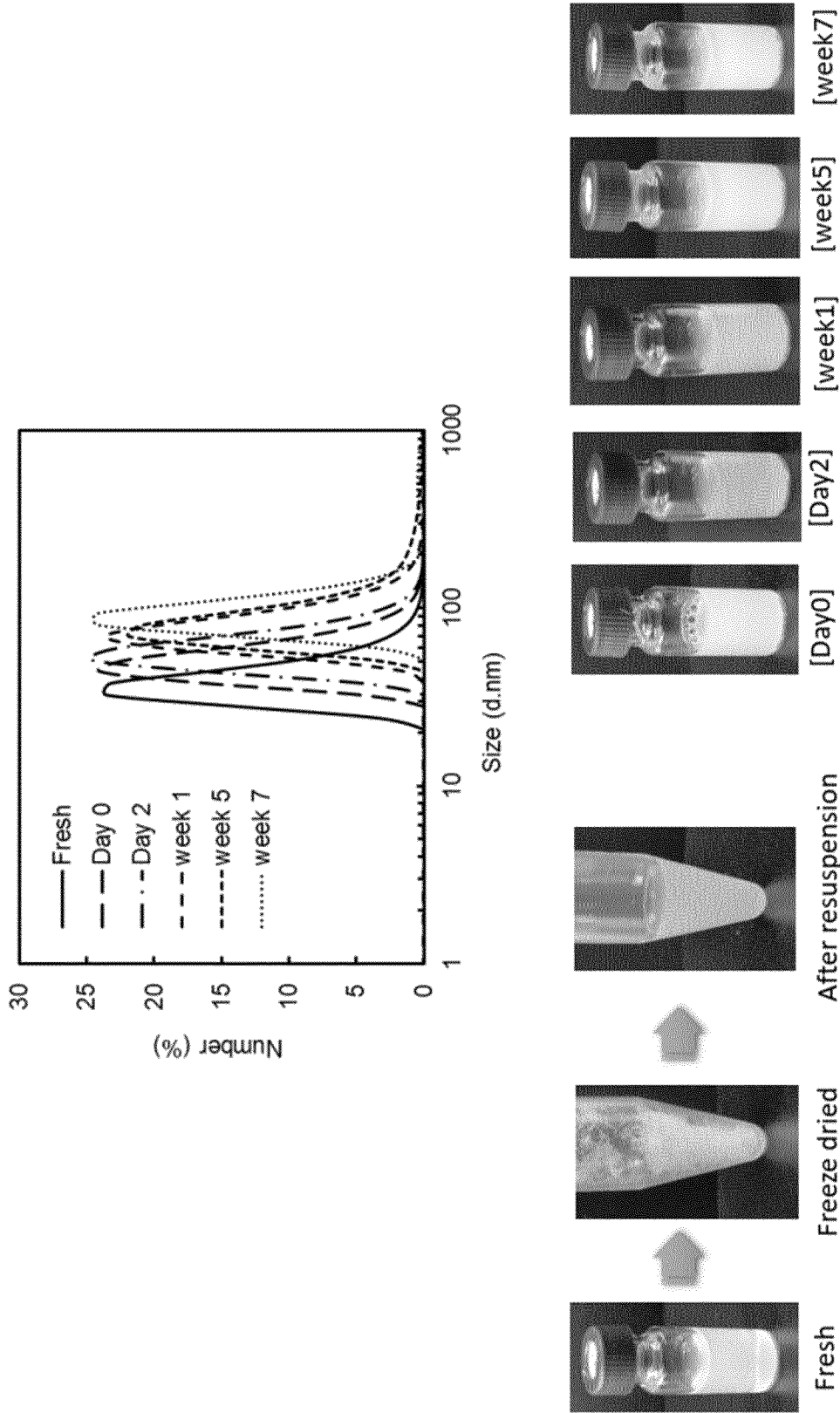


Fig. 13

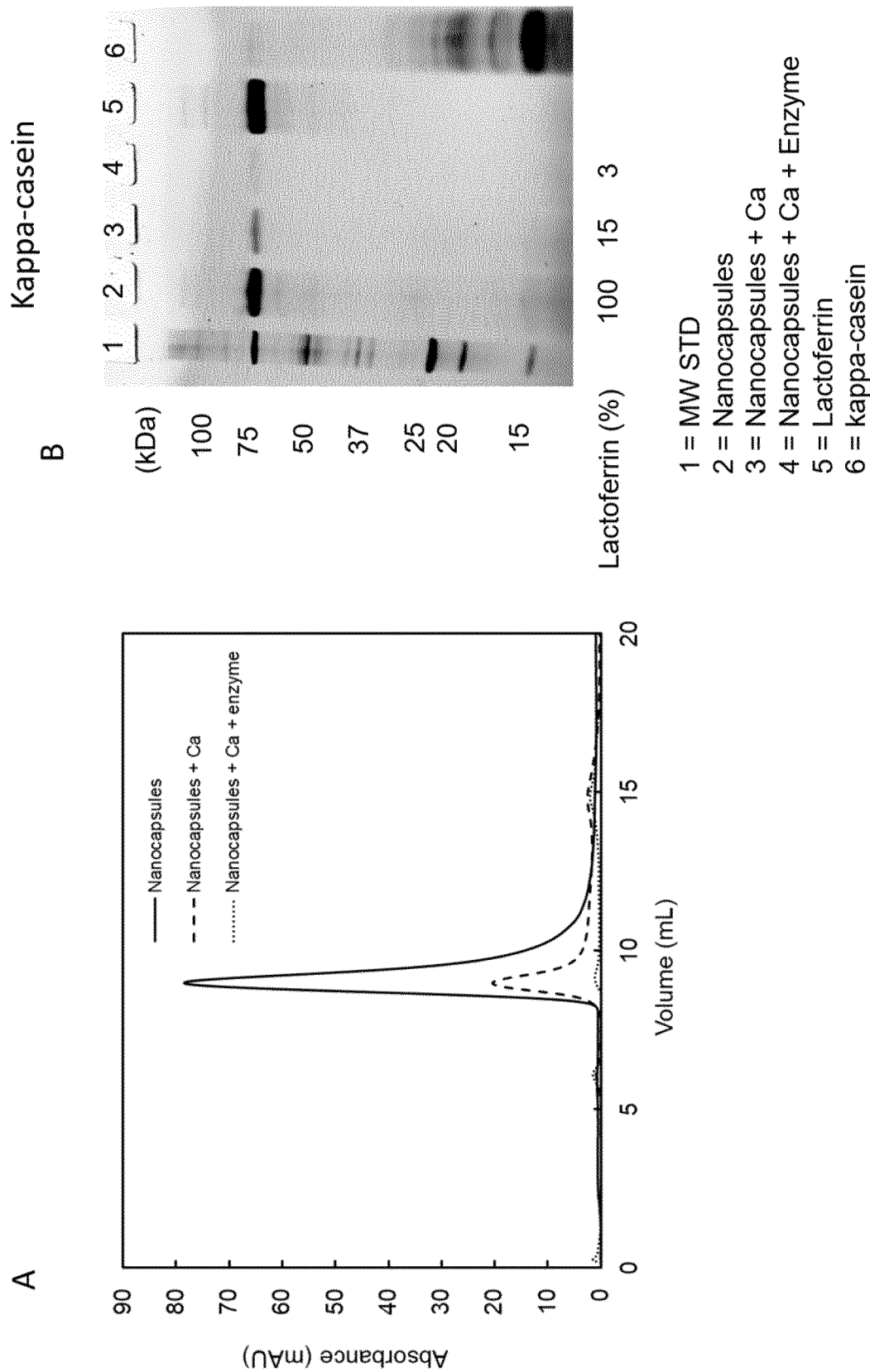


Fig. 14

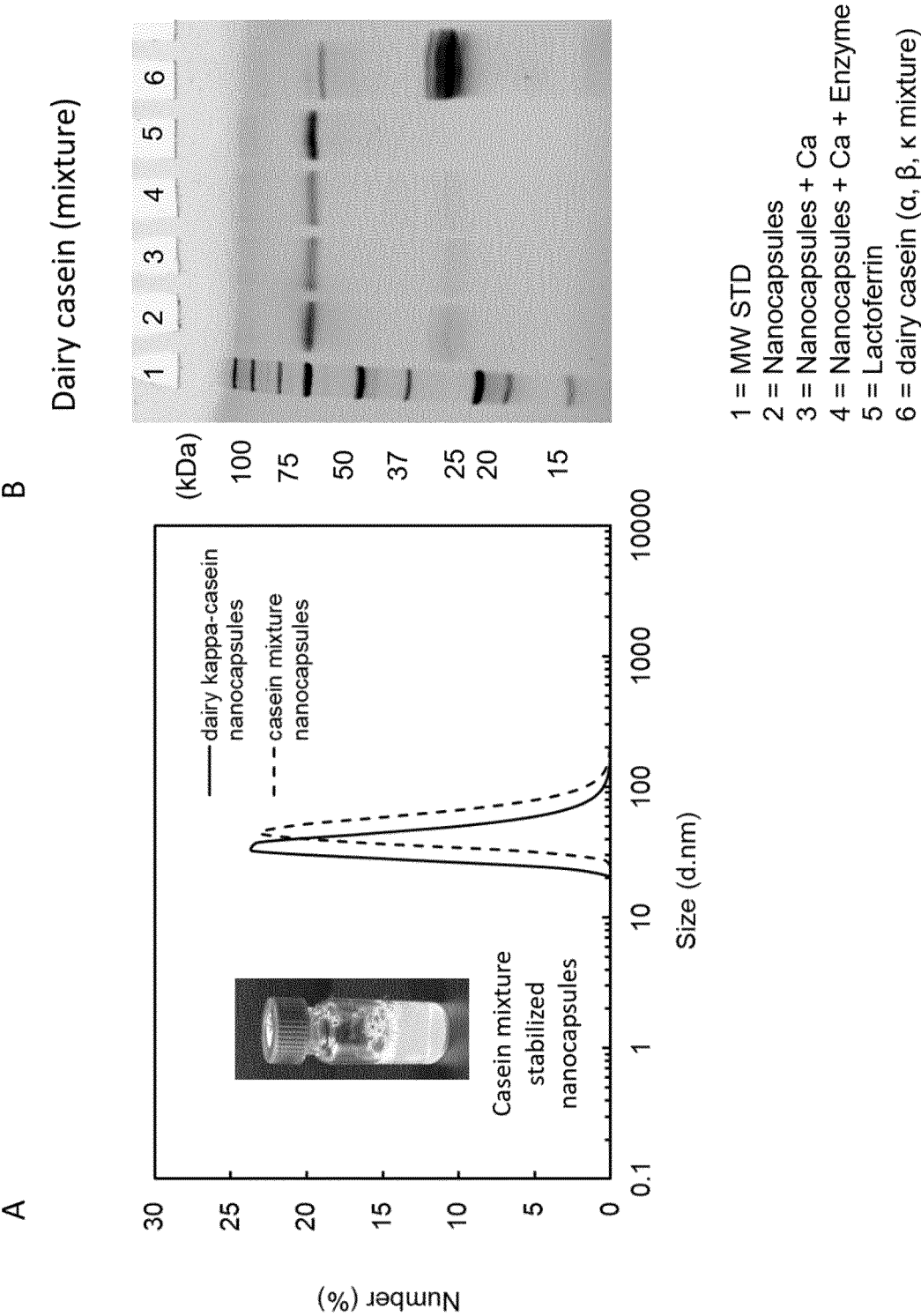


Fig. 15

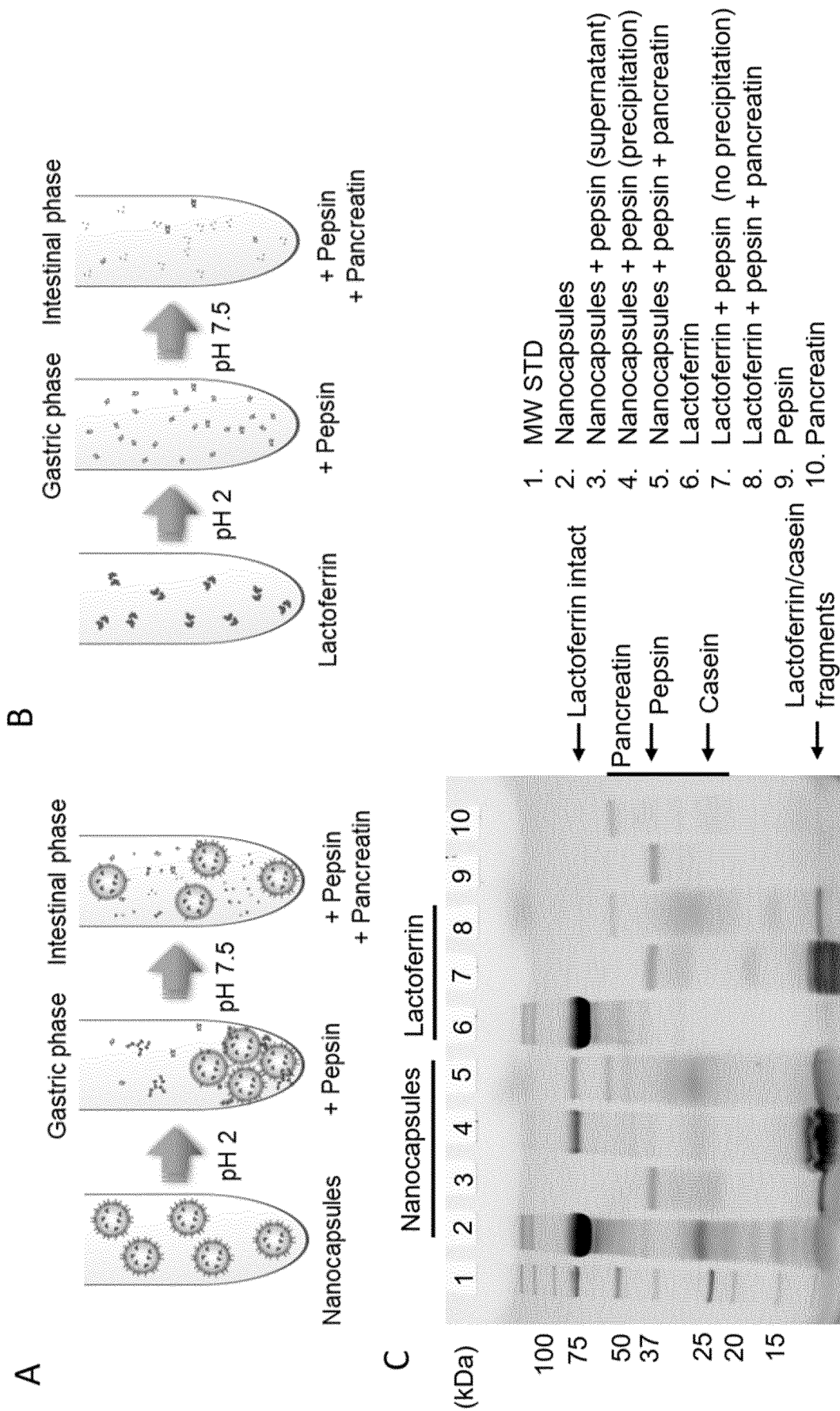


Fig. 16

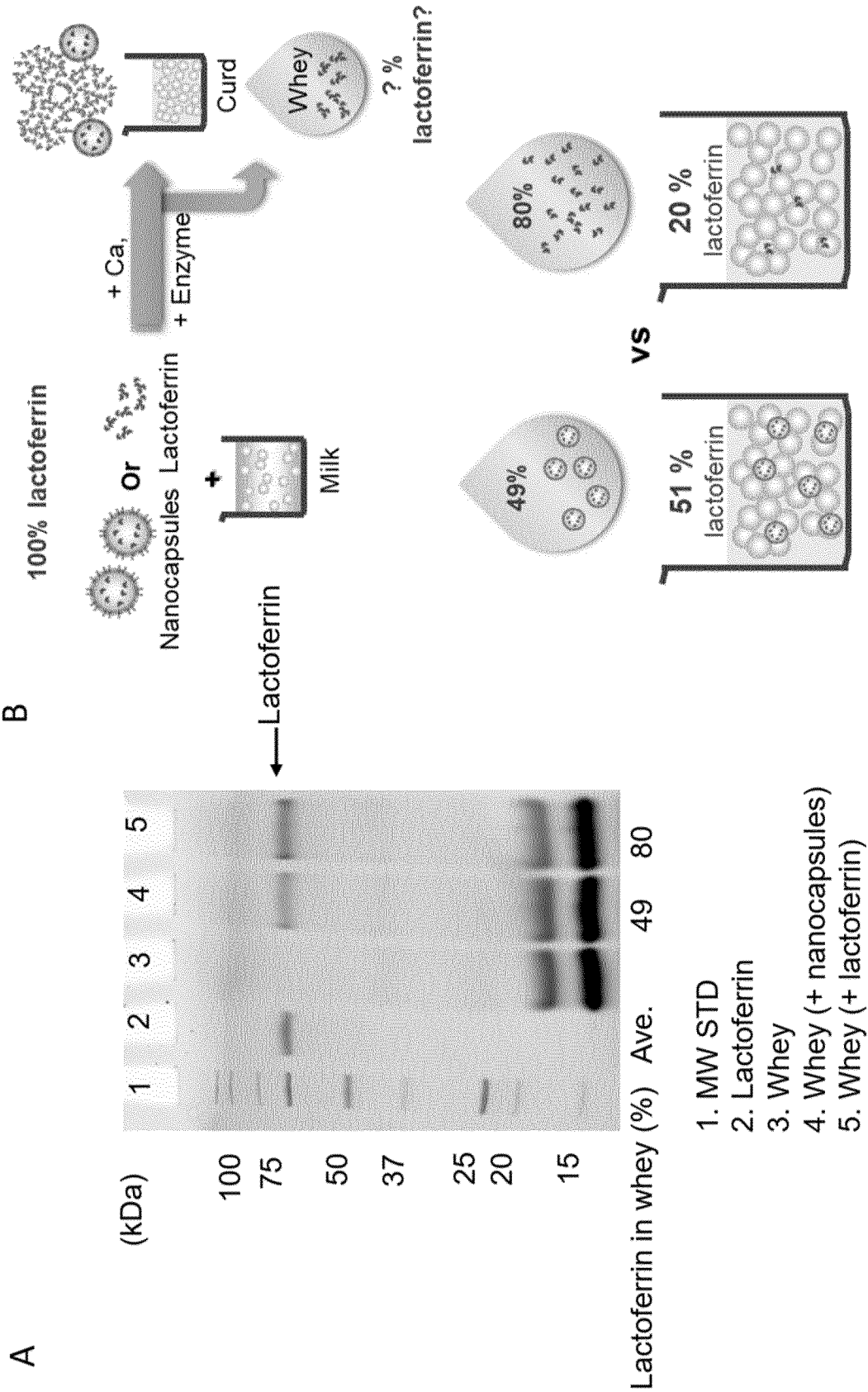


Fig. 17

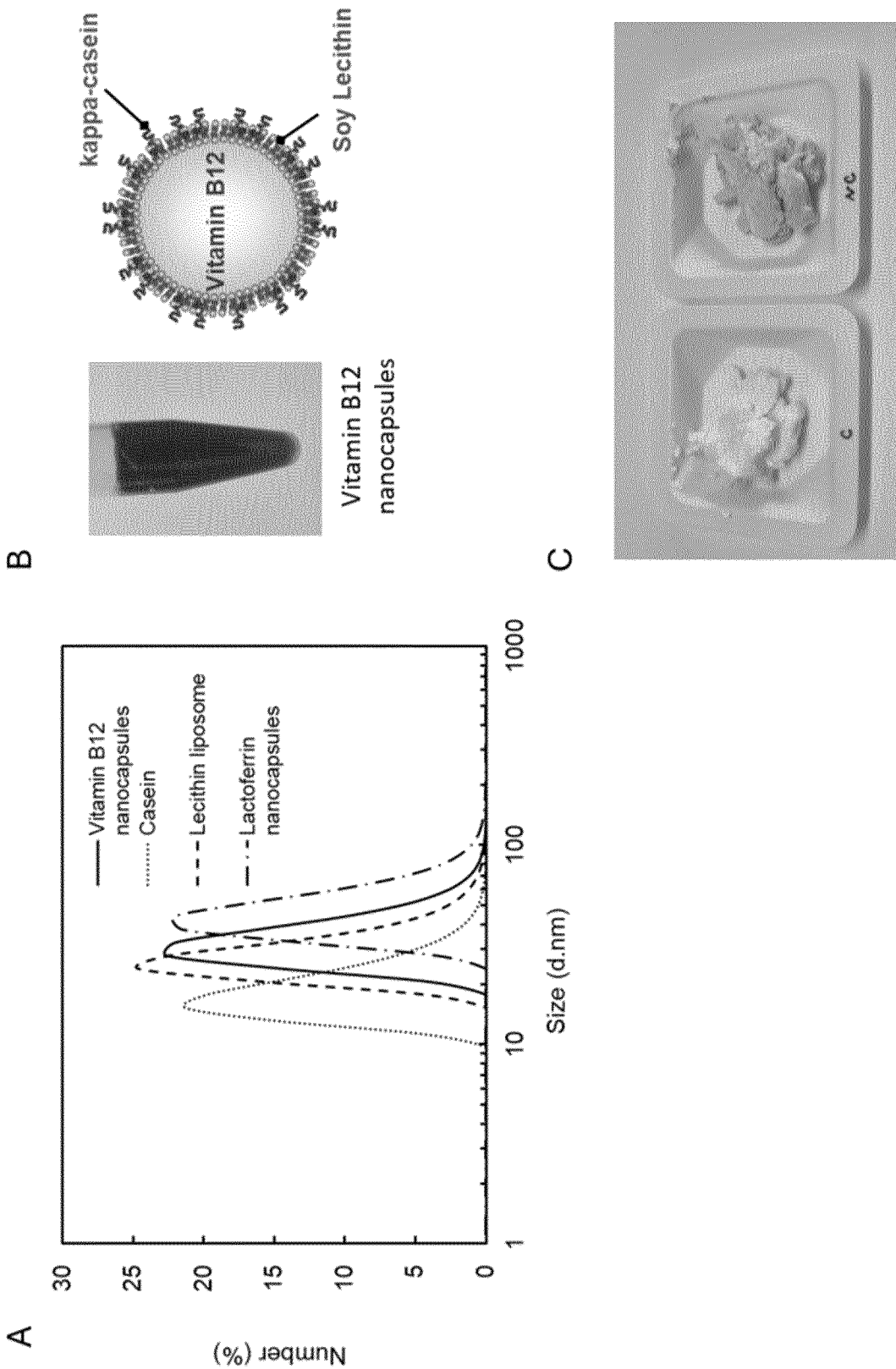


Fig. 18

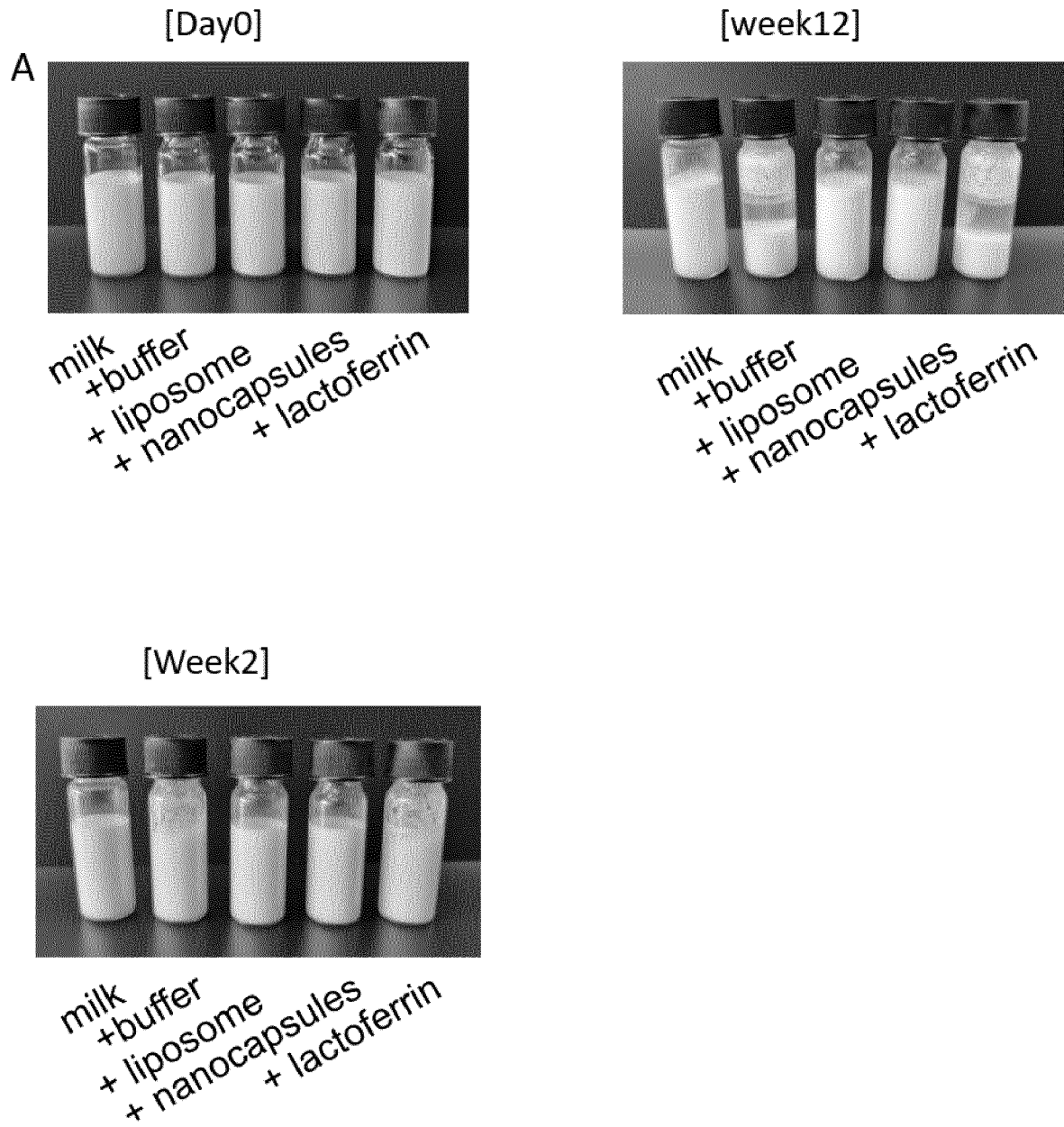


Fig. 19

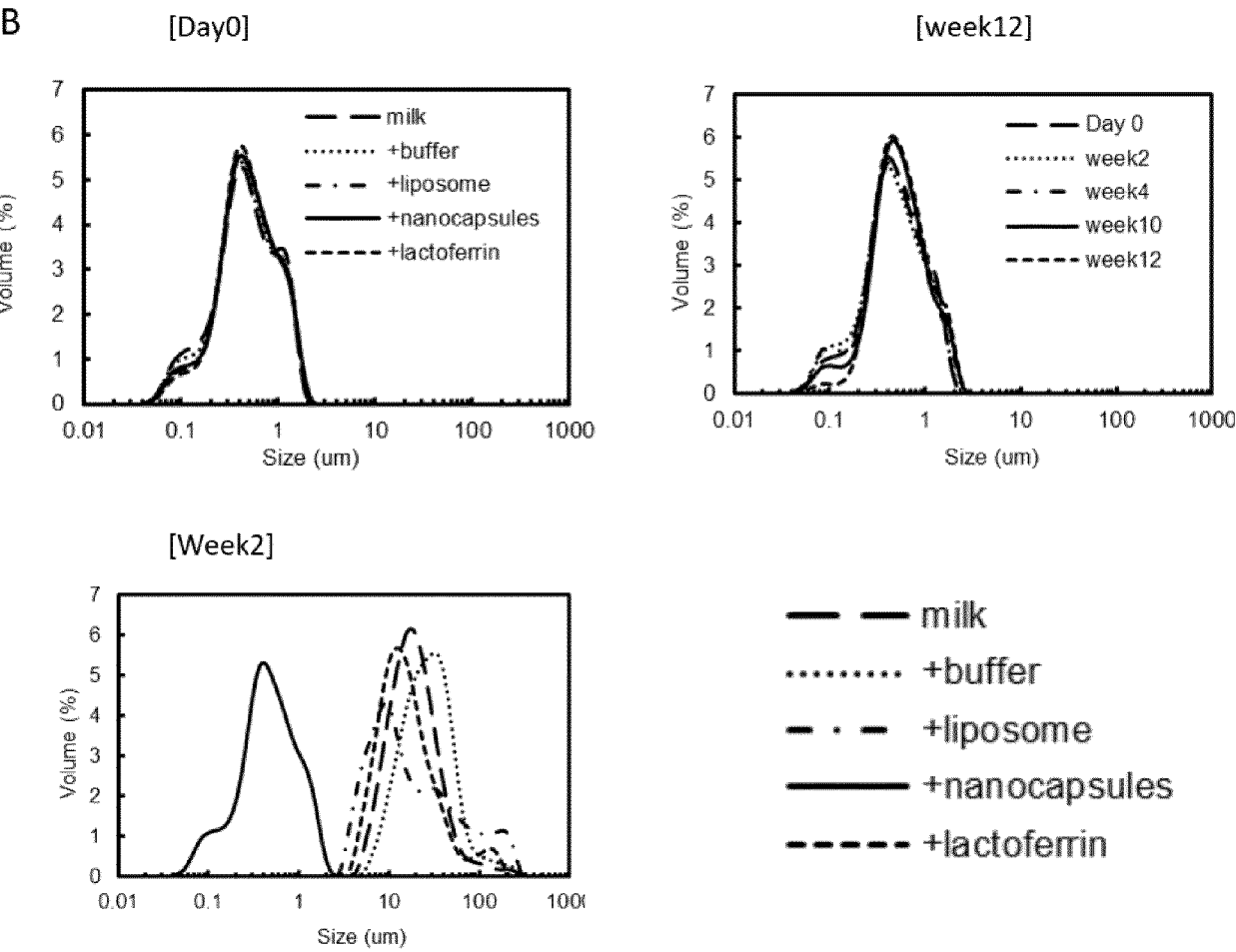


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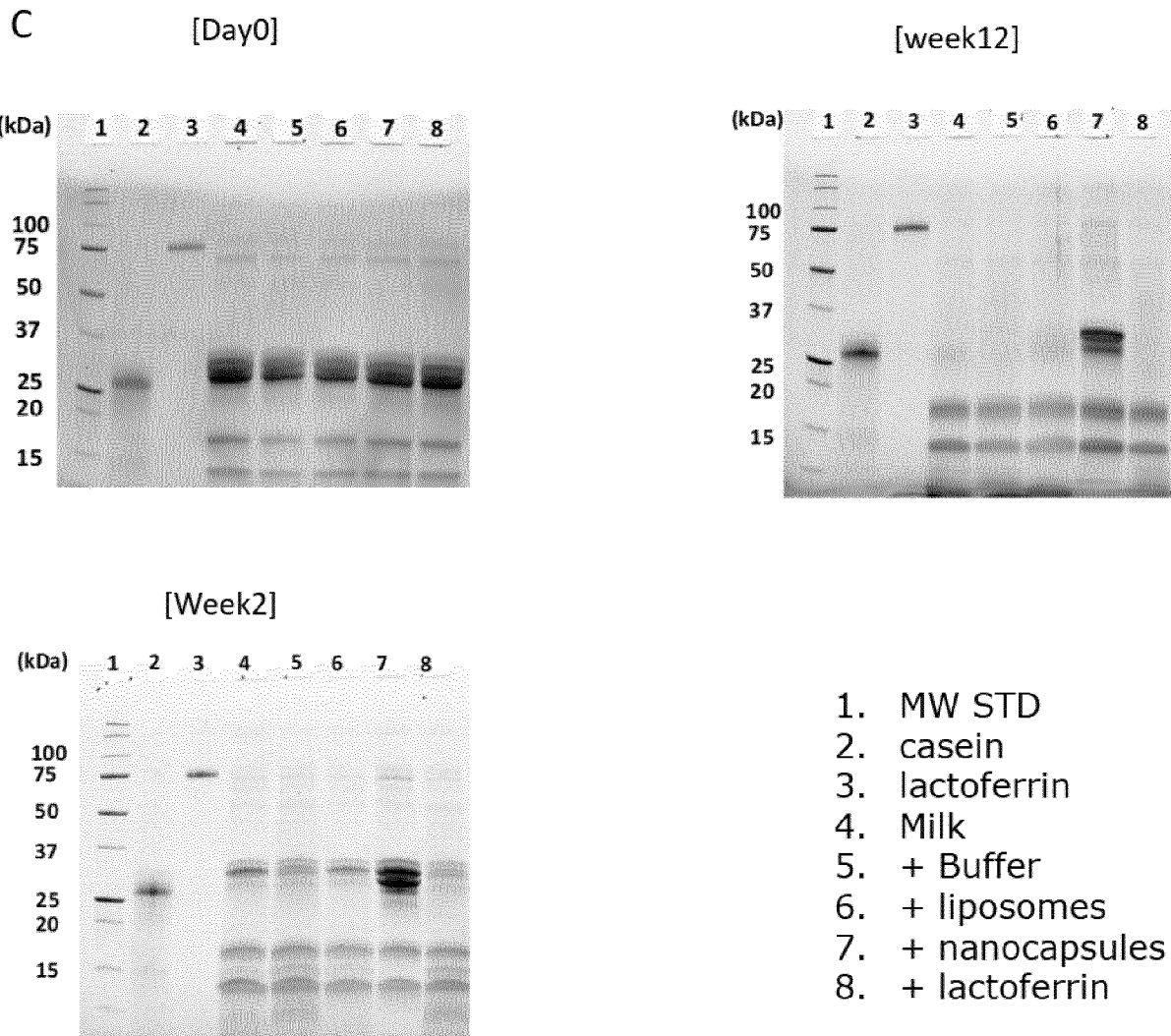


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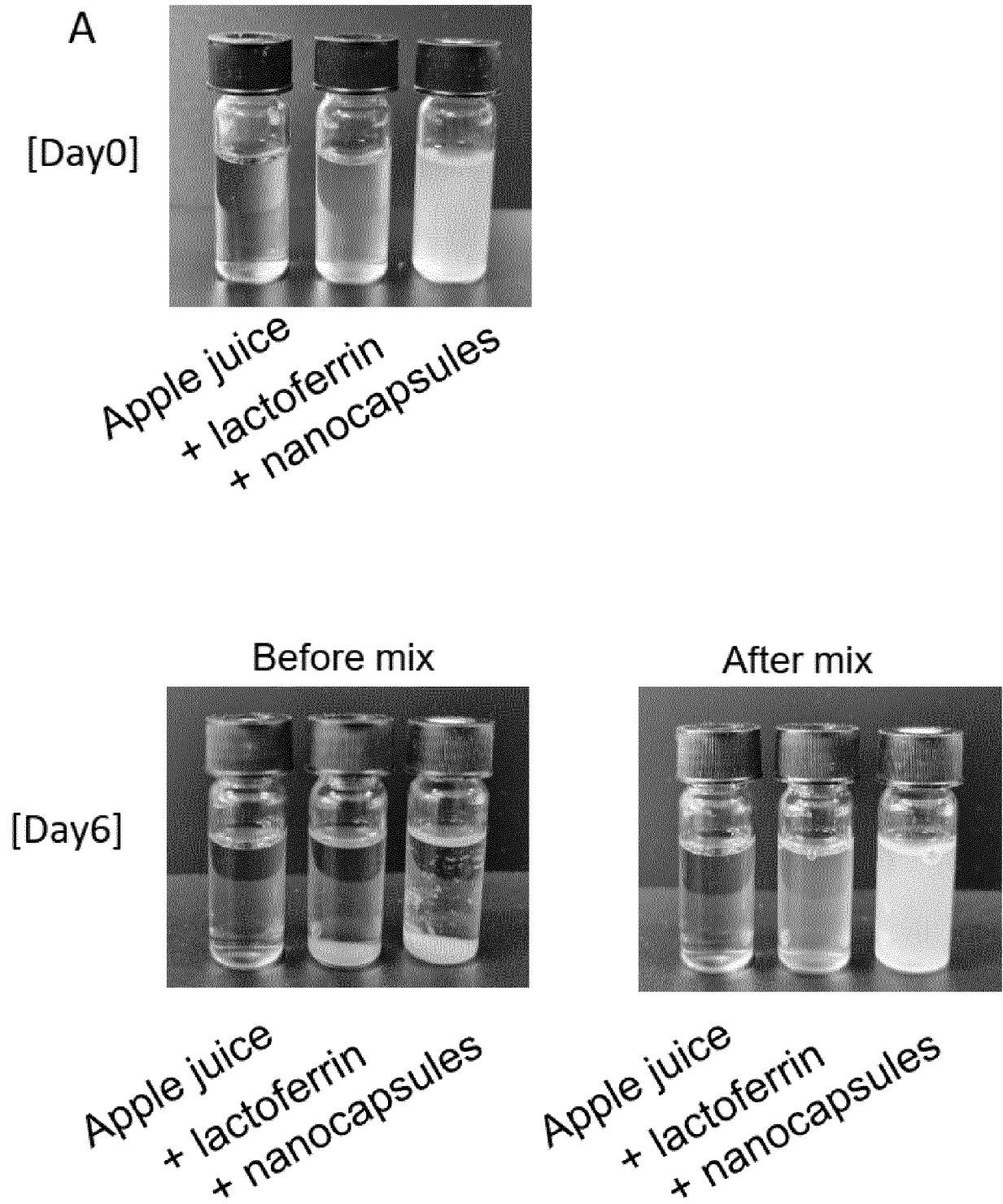
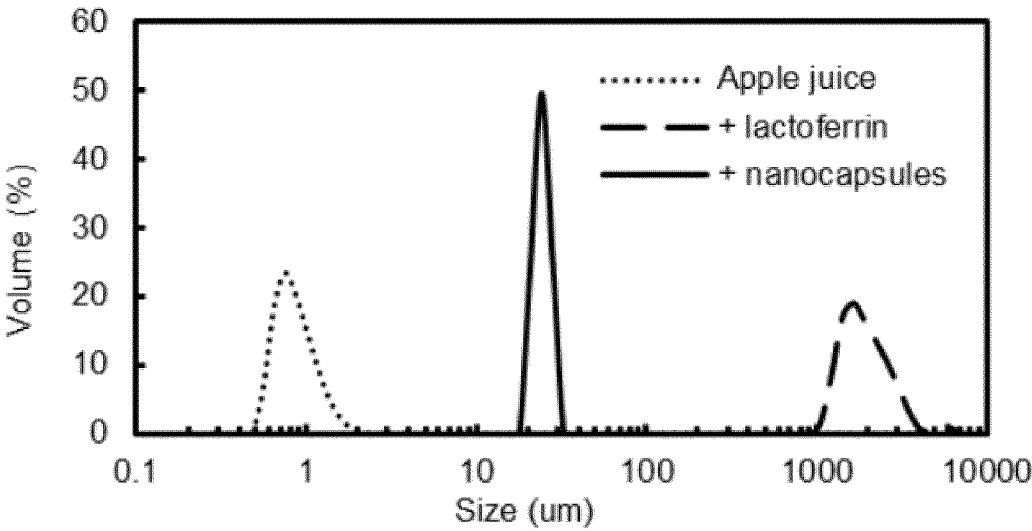


Fig. 20

B

[Day0]



[Day6]

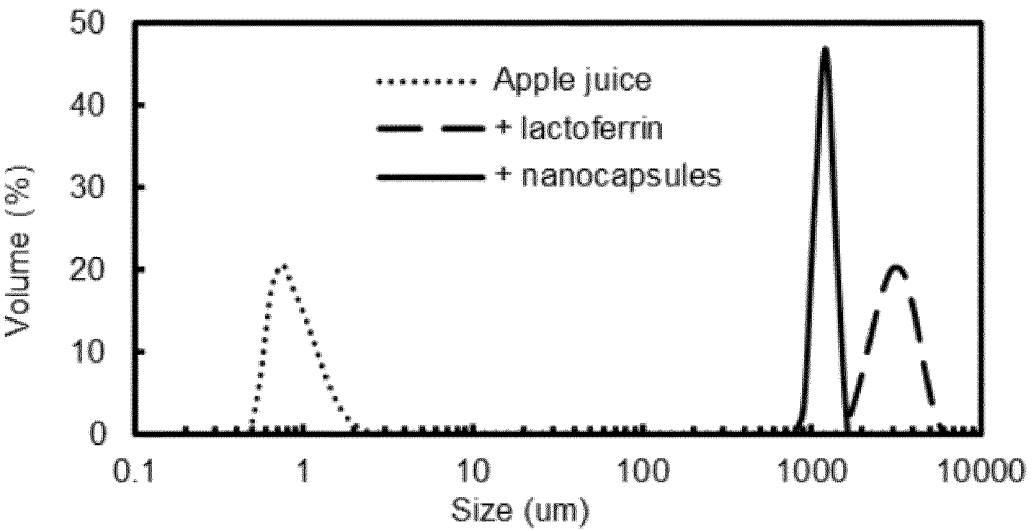


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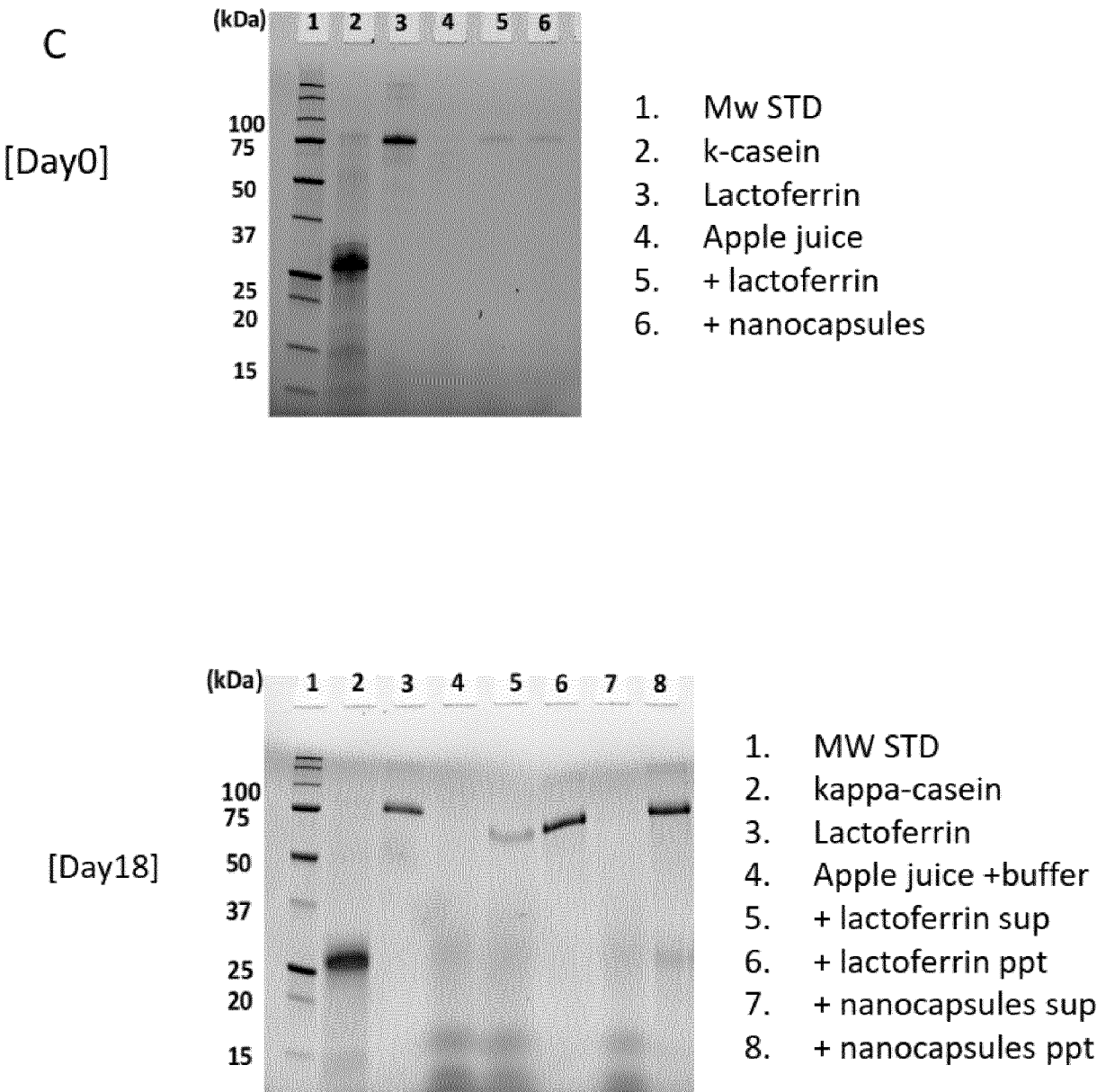


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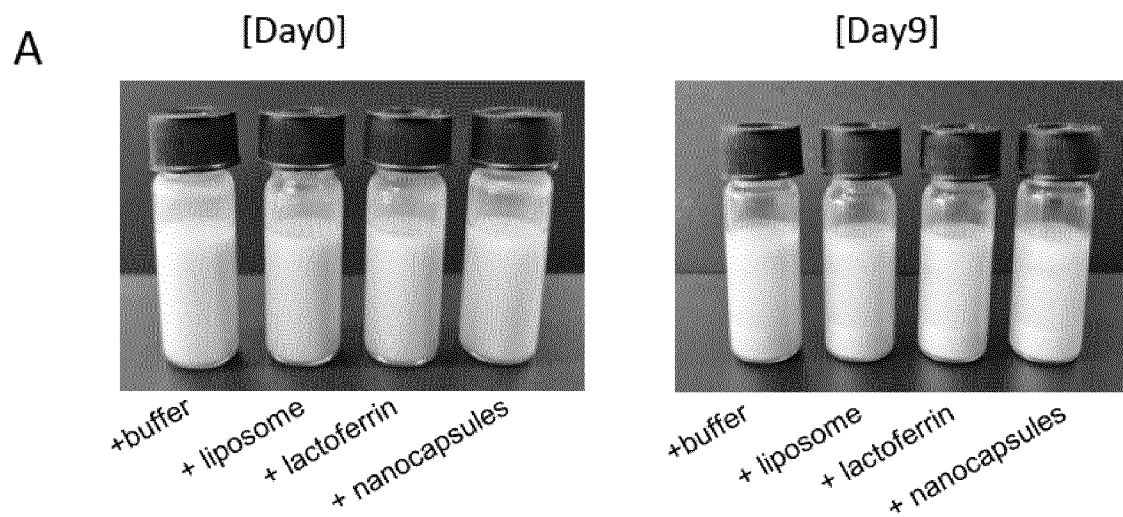


Fig. 21

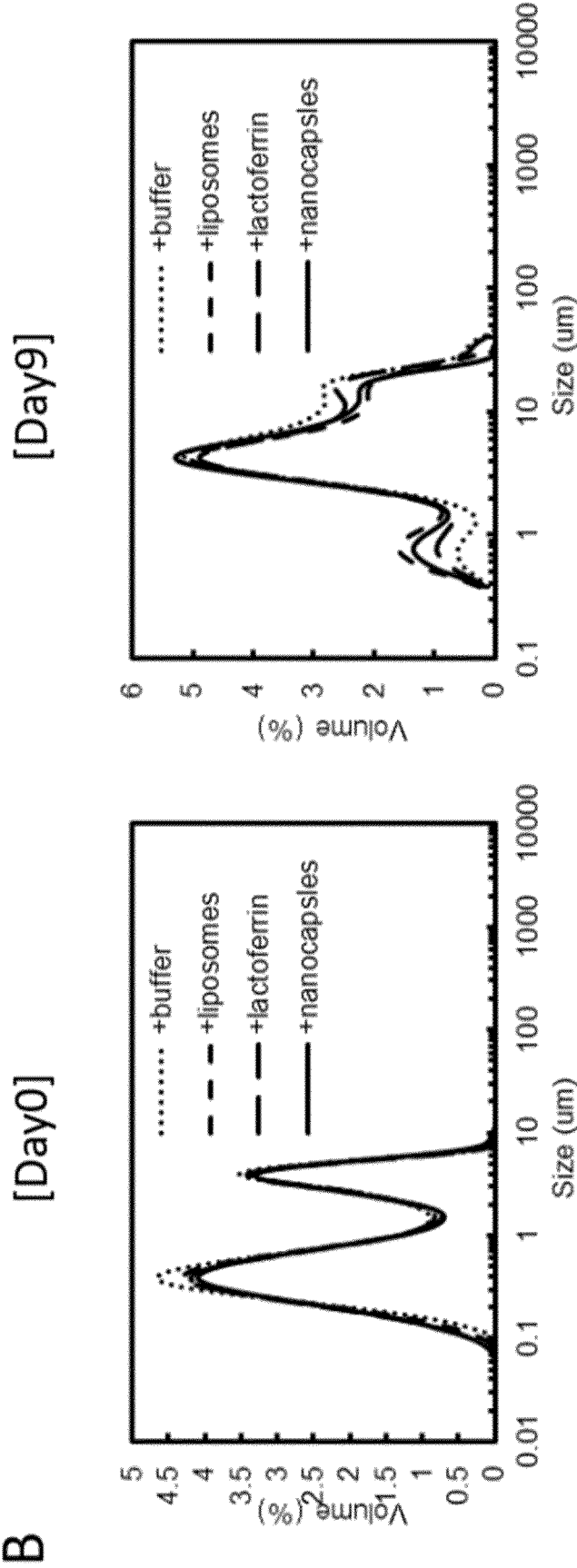


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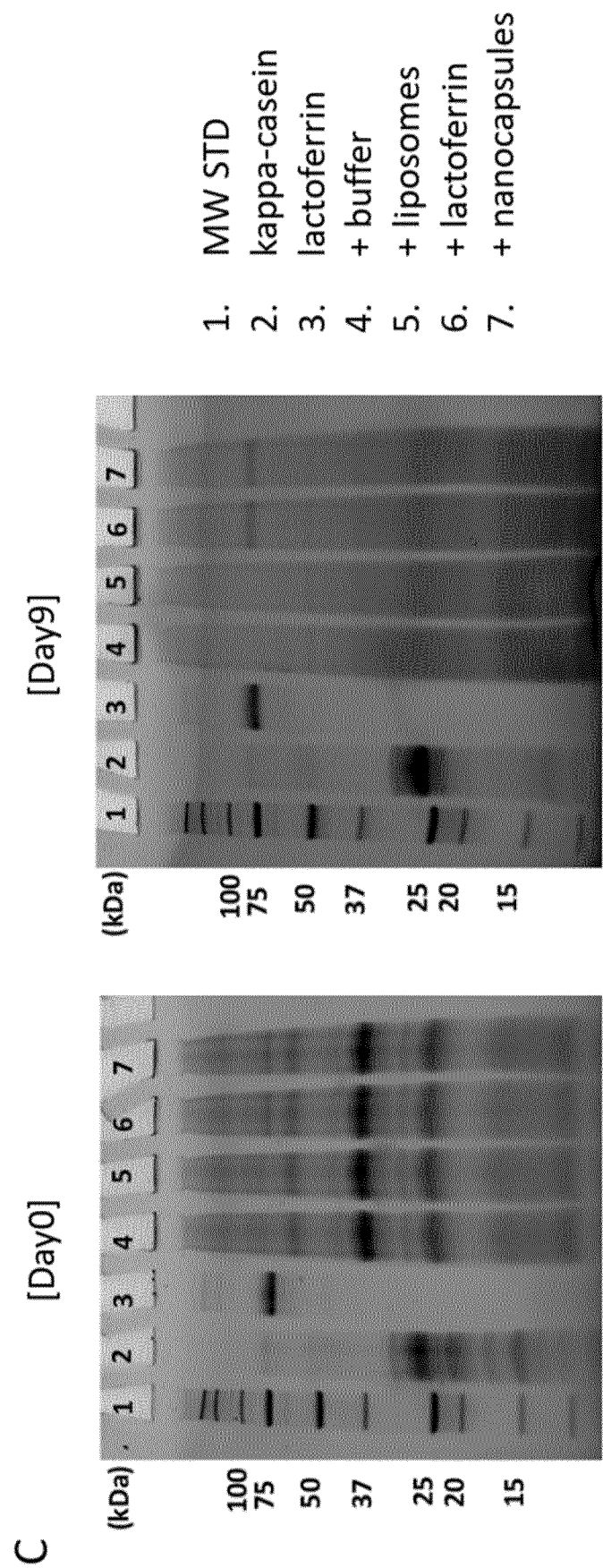


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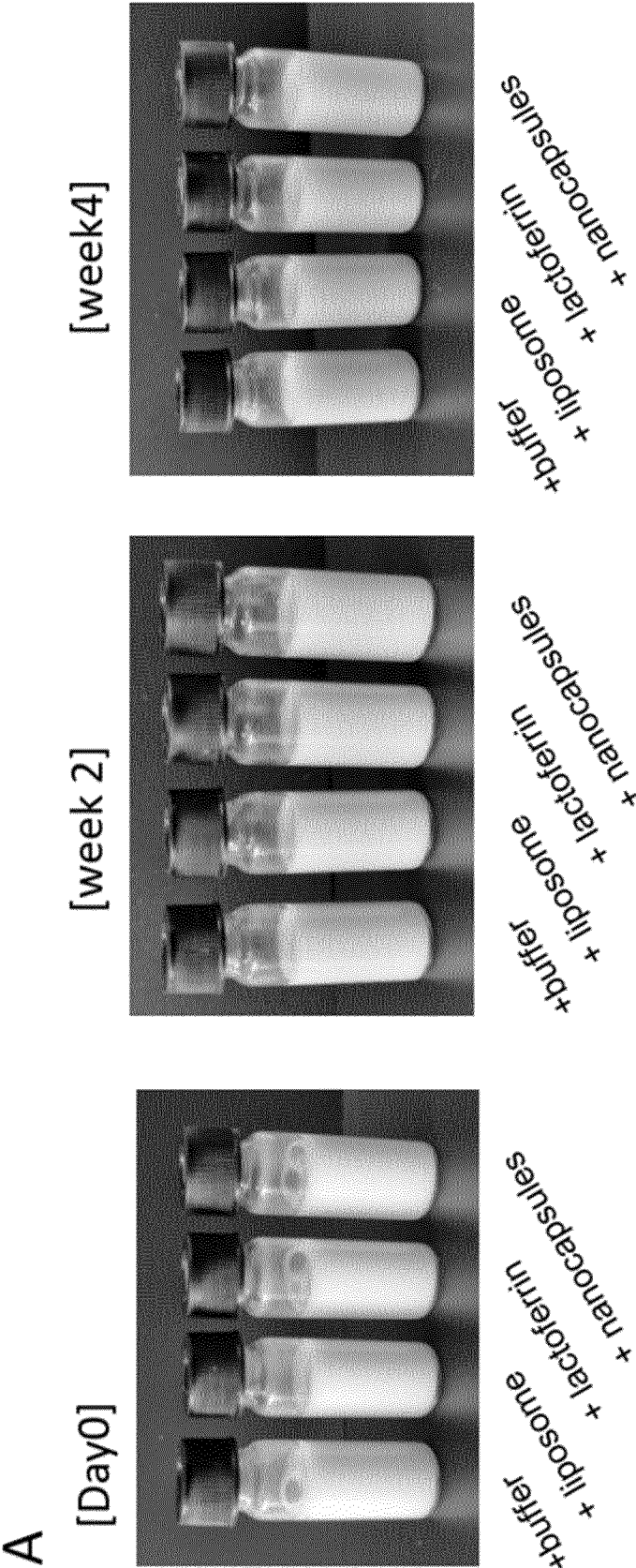


Fig. 22

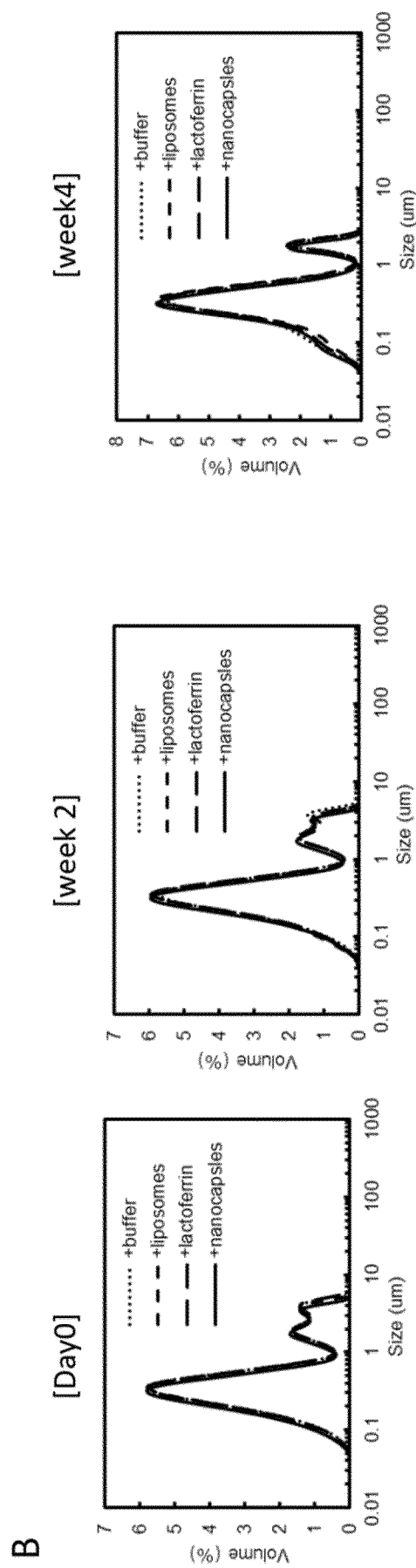


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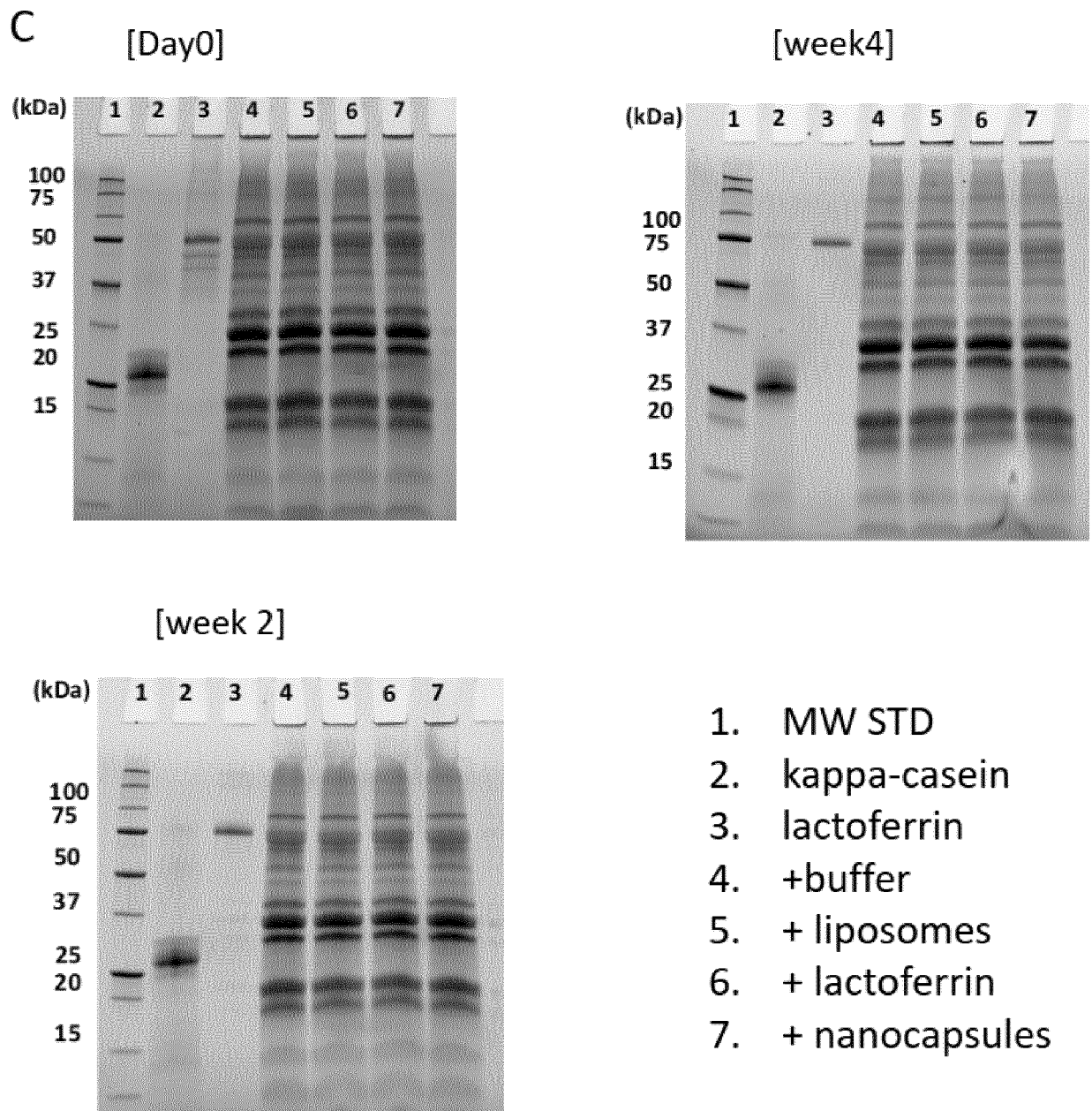


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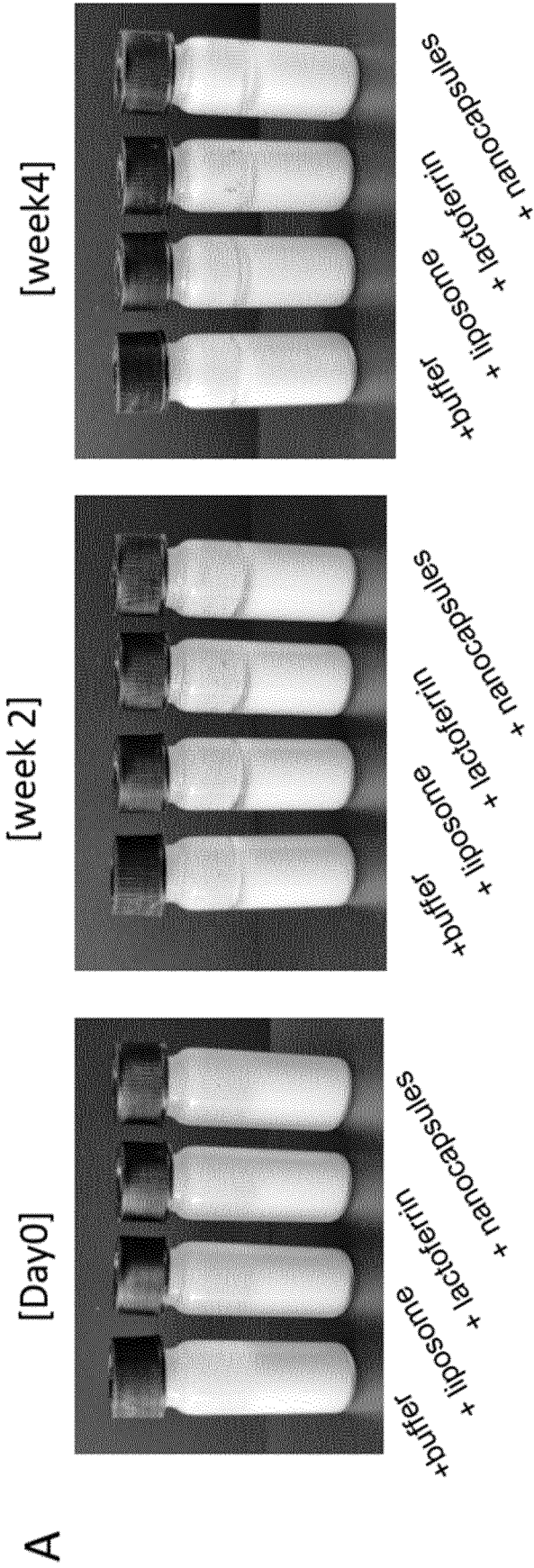


Fig. 23

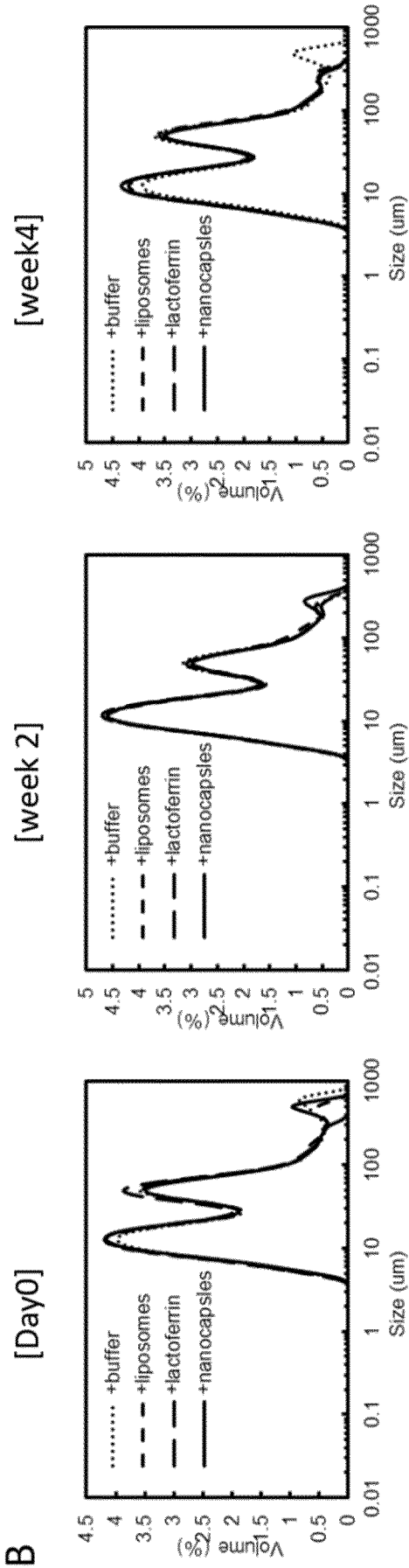


Fig. 23 cont.

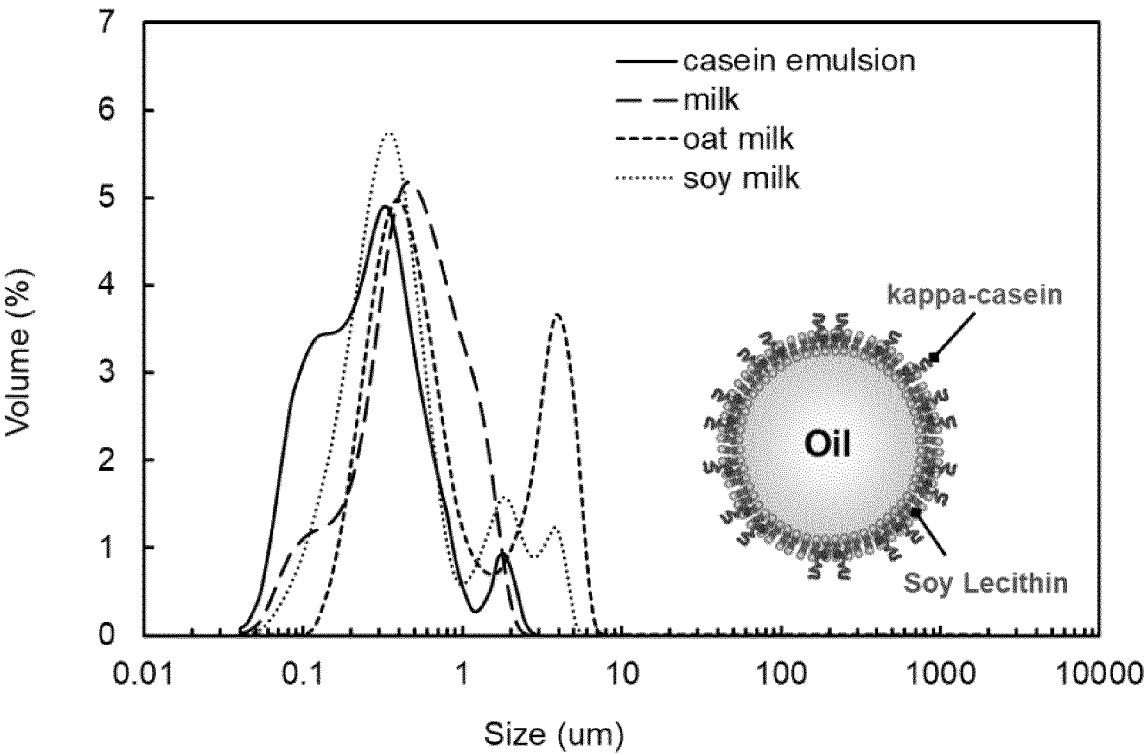


Fig. 24

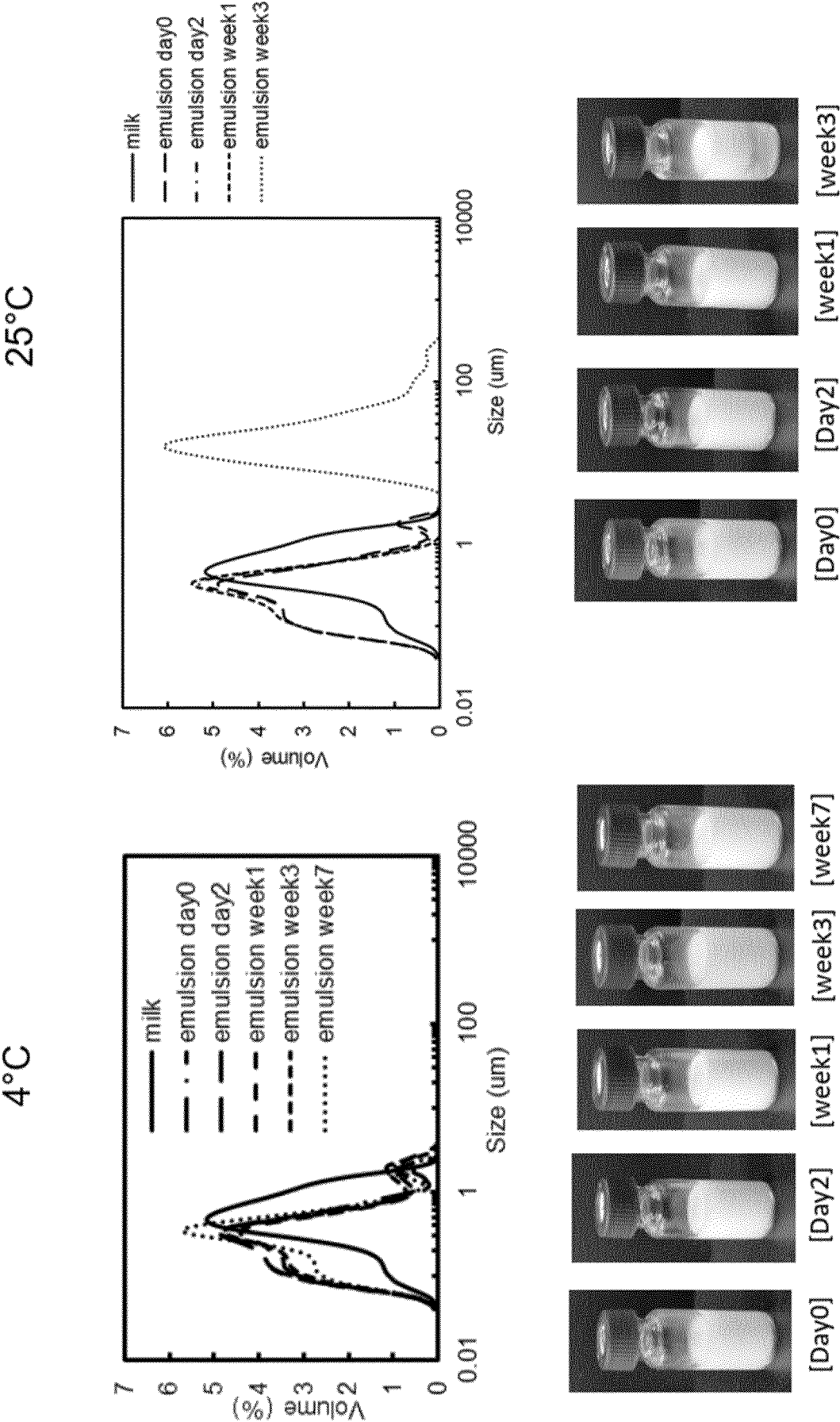


Fig. 25

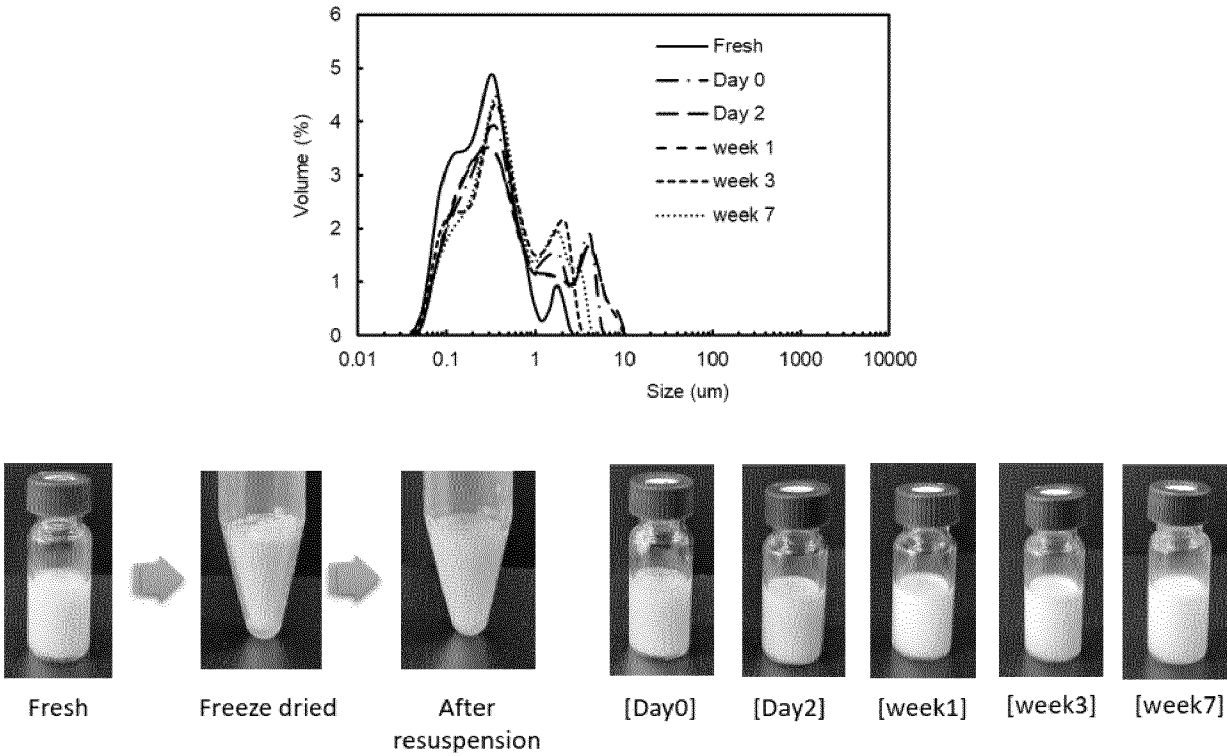


Fig. 26

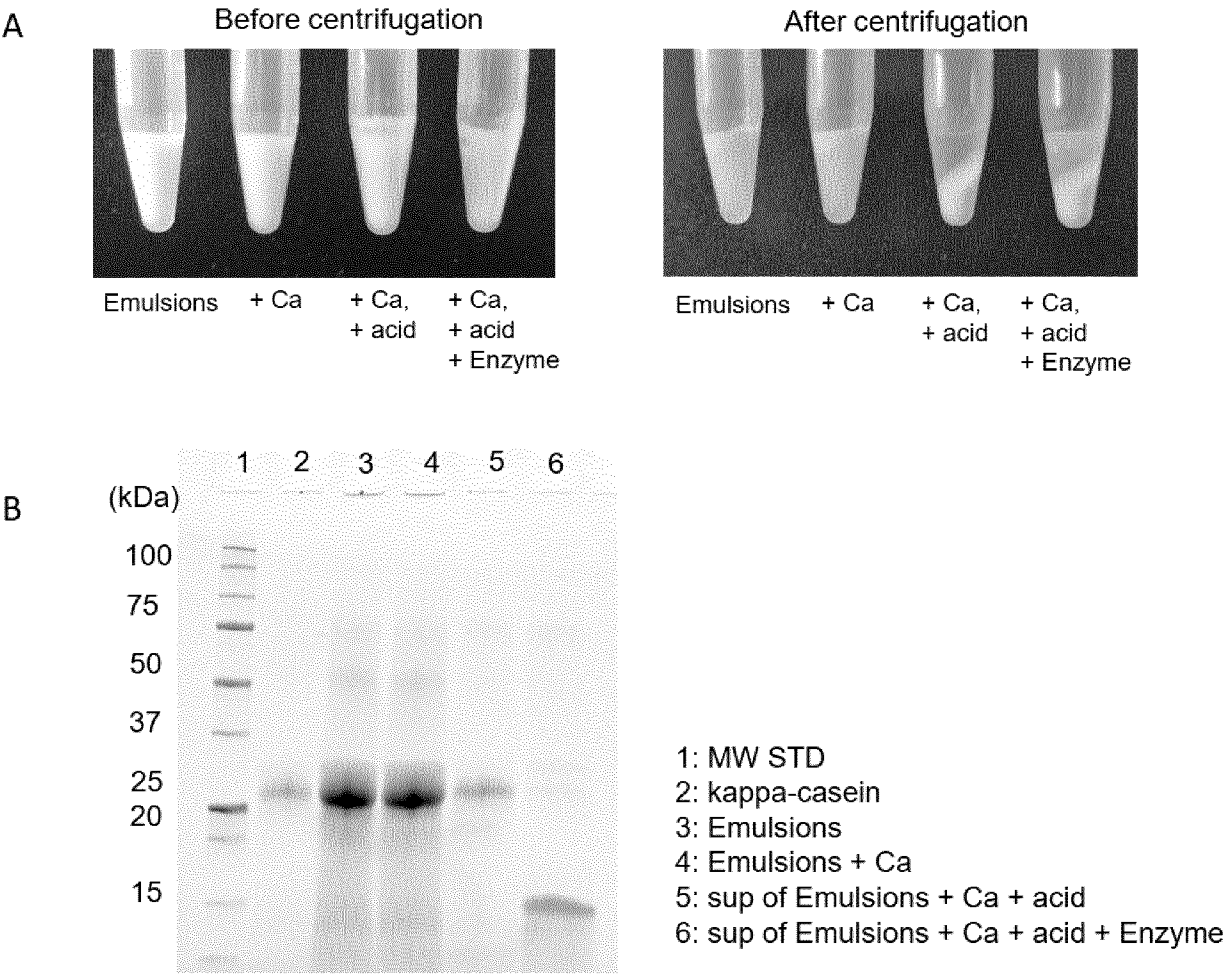


Fig. 27

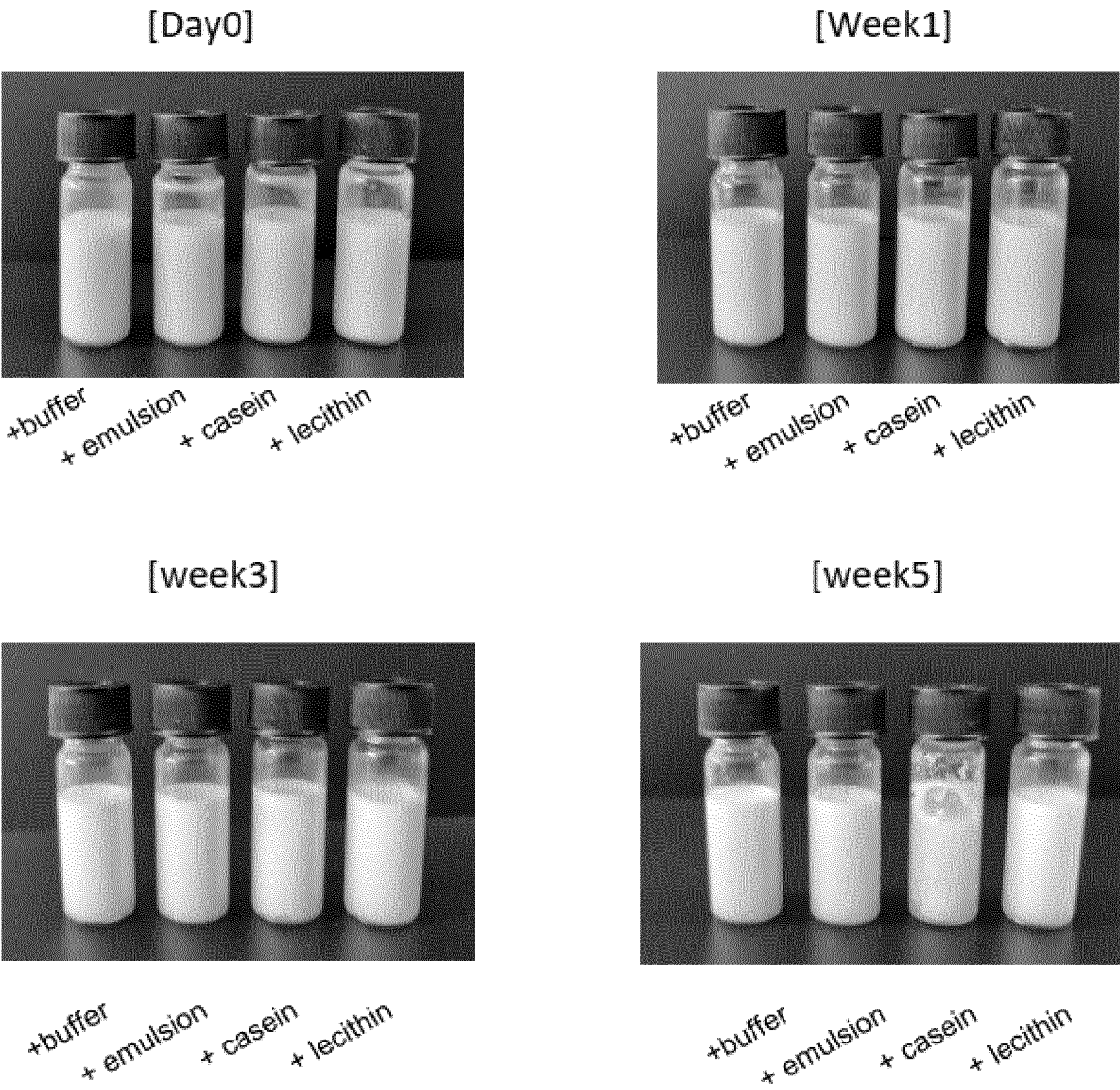


Fig. 28

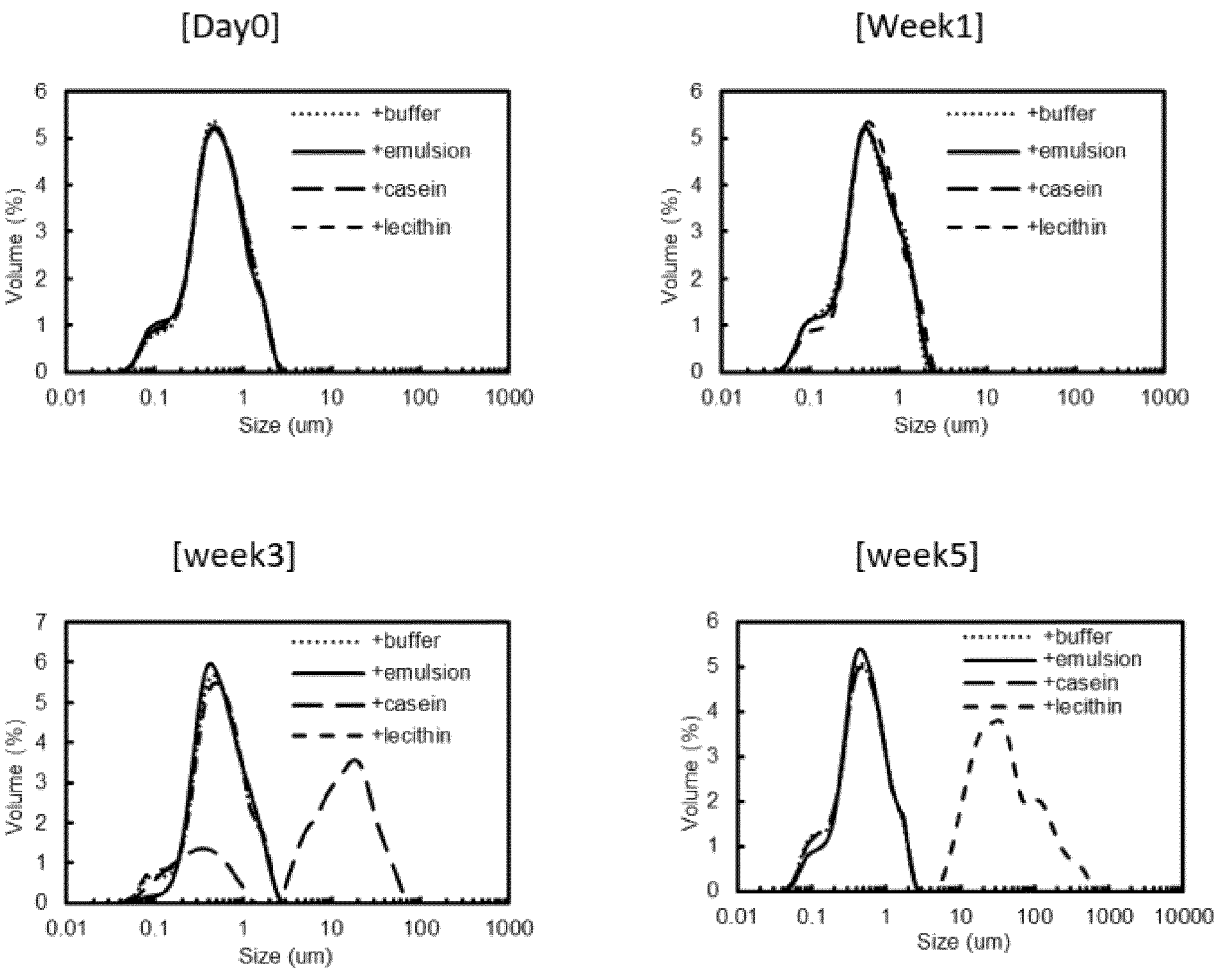


Fig. 28 cont.

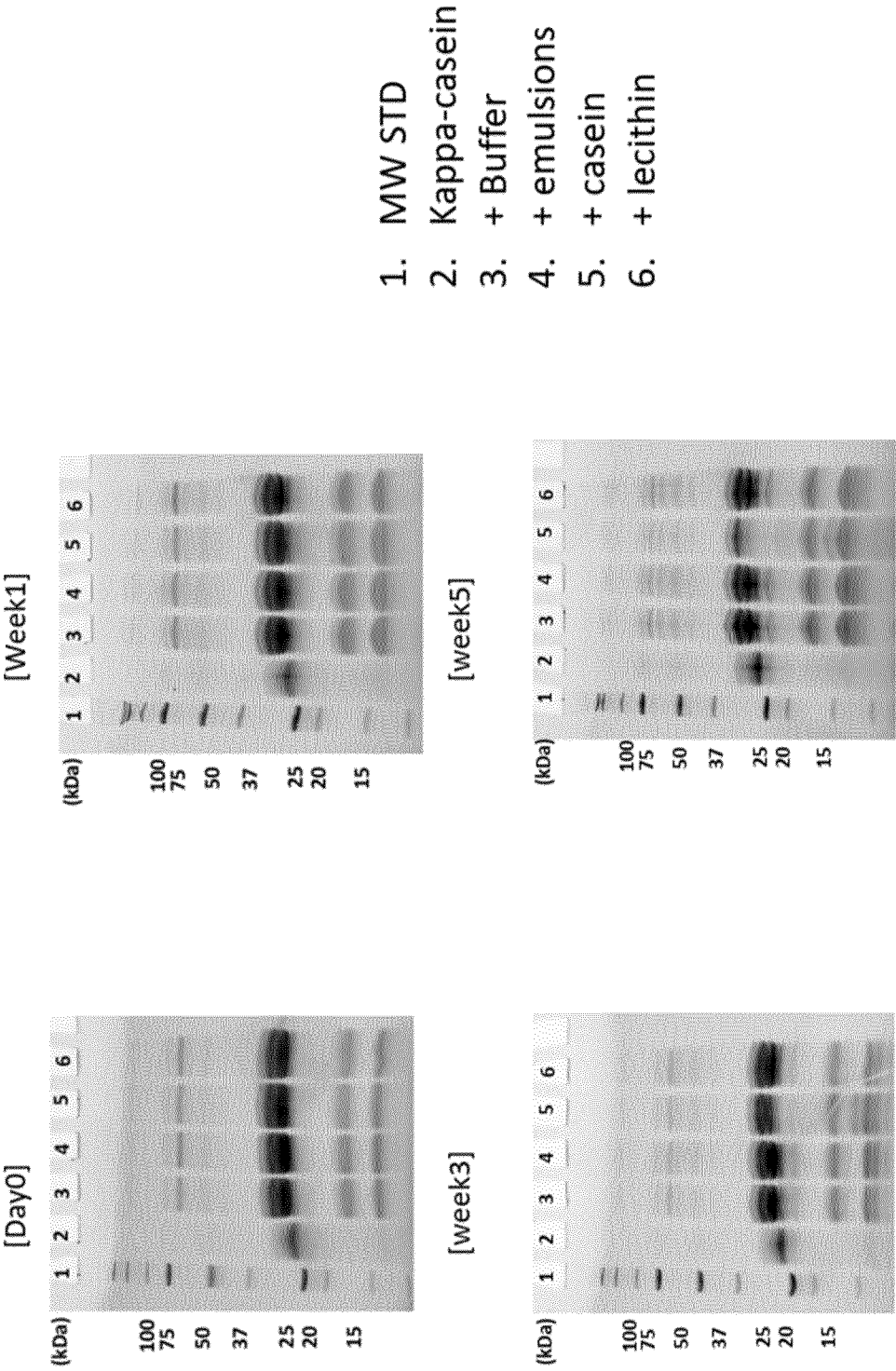


Fig. 28 cont.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2024/066610

A. CLASSIFICATION OF SUBJECT MATTER		
INV.	A23C19/05	A23L29/00
	A23P10/30	A23P10/35
ADD.	A23L29/10	A23L33/15
		A23L33/19
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)		
A23C A23L A23P		
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		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2019/254302 A1 (ABBASPOURRAD ALIREZA [US] ET AL) 22 August 2019 (2019-08-22) the whole document	2, 14, 15, 18-23
A	US 2010/151037 A1 (JIANG YIVAN [US] ET AL) 17 June 2010 (2010-06-17) the whole document	2, 14, 15, 18-23
A	US 2010/278975 A1 (CHUNG MYUNG JUN [KR] ET AL) 4 November 2010 (2010-11-04) the whole document	2, 14, 15, 18-23
A	CN 113 875 991 A (UNIV SHENYANG AGRICULTURAL) 4 January 2022 (2022-01-04) the whole document	2, 14, 15, 18-23
	- / - -	
☒ 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		Date of mailing of the international search report
23 September 2024		02/10/2024
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 Piret-Viprey, E

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2024/066610

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GUPTA UJALA ET AL: "Applications of lipid-engineered nanoplateforms in the delivery of various cancer therapeutics to surmount breast cancer", JOURNAL OF CONTROLLED RELEASE, ELSEVIER, AMSTERDAM, NL, vol. 348, 22 July 2022 (2022-07-22), pages 1089-1115, XP087138472, ISSN: 0168-3659, DOI: 10.1016/J.JCONREL.2022.05.034 [retrieved on 2022-07-22] the whole document -----	2,14,15, 18-23
A	RANADHEERA C S ET AL: "Utilizing unique properties of caseins and the casein micelle for delivery of sensitive food ingredients and bioactives", TRENDS IN FOOD SCIENCE & TECHNOLOGY, vol. 57, November 2016 (2016-11), pages 178-187, XP029789119, ISSN: 0924-2244, DOI: 10.1016/J.TIFS.2016.10.005 the whole document -----	2,14,15, 18-23

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2024/066610

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed.
 - b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2024/066610

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: **1, 3-13, 16, 17 (completely); 18-23 (partially)**
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims;; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.2

Claims Nos.: 1, 3-13, 16, 17(completely); 18-23(partially)

The present application contains 23 claims, of which 9 are independent. There is no clear distinction between the independent claims because of overlapping scope. There are so many independant claims, and they are drafted in such a way that the claims as a whole are not in compliance with the provisions of clarity and conciseness of Article 6 PCT, as it is particularly burdensome for a skilled person to establish the subject-matter for which protection is sought. The non-compliance with the substantive provisions is to such an extent, that the search was performed taking into consideration the non-compliance in determining the extent of the search (PCT-EPO Guidelines B-VIII, 3).

The claims are as follows:

- there are 3 independant product claims: claim 1 reciting a dispersion, claim 2 reciting a nanocapsule and claim 3 reciting an emulsion;
- there is no claim reciting a method of producing the dispersion but 14 independant method claims:
 - * claim 14 reciting a method of producing the nanocapsules,
 - * claim 16 reciting a method of producing the emulsion,
 - * a method of producing a food product with the dispersion,
 - * a method of producing beverages with the dispersion,
 - * a method of producing a food product and beverages with the dispersion,
 - * a method of producing a food product with the nanocapsules,
 - * a method of producing beverages with the nanocapsules,
 - * a method of producing a food product and beverages with the nanocapsules,
 - * a method of producing a food product with the emulsion,
 - * a method of producing beverages with the emulsion,
 - * a method of producing a food product and beverages with the emulsion,
 - * a method of producing cheese or cheese analogue with the dispersion,
 - * a method of producing cheese or cheese analogue with the nanocapsules, and
 - * a method of producing cheese or cheese analogue with the emulsion.
- there are 2 independant use claims, but in fact theses 2 claims correspond to 18 independant use claims:
 - * use of the dispersion for the transport of added value compounds,
 - * use

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

of the dispersion for the encapsulation of added value compounds,
 * use
 of the dispersion for the transport and the encapsulation of added value compounds,
 * use of the nanocapsules or the transport of added value compounds,
 * use of the nanocapsules for the encapsulation of added value compounds,
 * use of the nanocapsules for the transport and the encapsulation of added value compounds,
 * use of the emulsion or the transport of added value compounds,
 * use of the nanocapsules for the encapsulation of added value compounds,
 * use of the nanocapsules for the transport and the encapsulation of added value compounds,
 * use of
 the dispersion for enrichment of a food product with an added value compound,
 * use of the dispersion for enrichment of beverages with an added value compound,
 * use of the dispersion for enrichment of a food product and beverages with an added value compound,
 * use of the nanocapsules for enrichment of a food product with an added value compound,
 * use of the nanocapsules for enrichment of beverages with an added value compound,
 * use of the nanocapsules for enrichment of a food product and beverages with an added value compound,
 * use of the emulsion for enrichment of a food product with an added value compound,
 * use of the emulsion for enrichment of beverages with an added value compound, and
 * use of the emulsion for enrichment of a food product and beverages with an added value compound.

There is no example of dispersion as defined in claim 1 in the application. Most of the examples of the application (examples 1-18) are directed to nanocapsules. Therefore the search was based on the subject-matter that, as far as can be understood, could reasonably be expected to be claimed later in the procedure:

- . a nanocapsule, namely claim 2;
- . a method of producing the nanocapsule, namely claims 14, 15 and 18 (partly);
- . use of the nanocapsules for the transport and/or the encapsulation of added value compounds, namely claim 19 (partly);
- . use of the nanocapsules for enrichment of a food product and/or beverages with an added value compound, namely claims 20 (partly) and 21 (partly);
- . a method of producing a food product and beverages with the nanocapsules, namely claim 22 (partly); and
- . a method of producing cheese or cheese analogue with the nanocapsules, namely claim

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

23 (partly).

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guidelines C-IV, 7.3), should the problems which led to the Article 17(2) PCT declaration be overcome.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2024/066610

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2019254302 A1	22-08-2019	NONE	
US 2010151037 A1	17-06-2010	NONE	
US 2010278975 A1	04-11-2010	DK 2235158 T3	24-03-2014
		EP 2235158 A1	06-10-2010
		JP 5396402 B2	22-01-2014
		JP 2011510624 A	07-04-2011
		KR 20090082035 A	29-07-2009
		US 2010278975 A1	04-11-2010
		WO 2009093785 A1	30-07-2009
CN 113875991 A	04-01-2022	NONE	