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(54) **Title**: CALCIUM SENSITIVE GELATIN GELS

(57) **Abstract**: The invention provides a method for the production of a gel-based product, the method comprising (i) providing a liquid composition comprising gelatin in an amount of 0.1-30 wt.%, and (ii) adding a calcium salt to the liquid composition to provide a calcium concentration in the liquid composition up to 50 m M, wherein when combining the gelatin and calcium salt the liquid composition substantially does not comprises a cross-linker,and wherein the gelatin comprises amino acids with at least 1% of the total amount of amino acids being succinylated or phtalated.

Calcium sensitive gelatin gels

FIELD OF THE INVENTION

The invention relates to a method for the production of a gel-based product. The invention further relates to a product obtainable with such method, such as a food or non-food product. The invention also relates to the use of functionalized gelatin.

BACKGROUND OF THE INVENTION

The use of gelatin in food and non-food applications is well known in the patent and non-patent art. WO2009153750, for instance, describes compositions comprising a cross-linkable protein or polypeptide, and a non-toxic material which induces cross-linking of the cross-linkable protein. The compositions are prepared in a non-phosphate buffer solvent. Optionally and preferably, the cross-linkable protein includes gelatin and any gelatin variant or variant protein as described herein. Optionally and preferably, the non-toxic material comprises transglutaminase (TG), which may optionally comprise any type of calcium dependent or independent transglutaminase, which may for example optionally be a microbial transglutaminase (mTG). Further, by way of example of a food application, US5232727 describes a frozen food produced by adding a gelling agent comprising gelatin to a dough mainly consisting of wheat flour, fat and egg. The process for producing the frozen food comprises adding a gelling agent comprising gelatin to dough materials mainly consisting of wheat flour, fat and egg to thereby give a dough and then molding and freezing said dough.

US3266906 describes a composition suitable for producing a high bloom strength alginate gel by incorporation with an aqueous medium comprising a water soluble alginate.

GB1474891 describes synthetic caviar comprises granules of an aqueous gel of gelatin containing edible proteins and enclosed in two pellicles, namely an inner pellicle consisting of the products of tanning the gel with a vegetable tannin and an outer pellicle containing a calcium and/or aluminum salt of an edible polysaccharide. Both pellicles and the gel may also contain ferric salts, and an edible eno or annatto dye may also be present in the gel.

EP1367066 describes a process for preparing a gelatin composition suitable for use in the preparation of a blood plasma extender, which process comprises: (a) pre-conditioning, under alkaline conditions, a gelatin-producing raw material; (b) preparing, from the pre-conditioned raw material product of step (a), a gelatin starting material having a pl (iso-ionic point) in the range of from 4.5 to 6; and (c) derivatising the gelatin starting material product of step (b), whereby there is produced derivatised gelatin having a pl in the range of from 4 to 5. The derivatised gelatin thereby produced preferably has a pl in the range of from 4 to 4.8 and a degree of derivatisation in the range of 50-60%. A succinylated gelatin thereby produced is particularly suitable for use in the preparation of a blood plasma expansion products.

SUMMARY OF THE INVENTION

Collagen is an abundant protein organized in tissues with a highly ordered molecular axis in fibrous networks contributing to the spatial cellular structure. Gelatin is the product of structural and chemical degradation of collagen that still resembles most of the functional properties of the original molecule. As indicated above, gelatin is widely used in a variety of applications in different sectors of the food industry as bakery (to promote emulsification, or gelling and stabilization properties), dairy (stabilization and texture), meat products (water-binding), and confectionery as well as in specific products like jam, jelly and low-fat spreads (promoting creaminess, fat reduction and mouth feel). The versatility of gelatin in functional properties in production of foods makes it a valuable tool for developing new and more attractive or tailored products for consumers. One of the most important quality parameters in processed foods is the textural attribute. The importance of understanding the characteristics of microstructural element formation of gelatin networks and their interactions lies in the established relationship between sensorial perception (mouth-feel) of food-based protein gels and its structural building blocks. The gel structure has an important influence on the mechanical properties, which in turn is controlled by the mechanism of gel network-formation and mechanical breakdown. Moreover, gelatin may also be used in non-food applications, such as coatings or encapsulates of pharmaceuticals.

A primordial attribute of gelatin-gels is the ability to store energy in the network (elasticity) that can be recovered when an applied deformation relaxes. Profound stiffness of gelatin gels has been related to the presence of long triple helices connected by flexible strands. For fibrous networks, like that of gelatin, a number of structural

motifs seem to dictate the macroscopic functionality. These are: (i) the mesh size of the network as set by the average distance between junction zones, which is protein concentration dependent, (ii) the length of the dominant structural motif, like the triple helix in gelatins, and (iii) the thickness, and thereby inherent flexibility of the structural motifs. A disadvantage of functionalizing gelatin is that the gelling properties may change, or some properties may improve while other properties get worse. For instance, gel strength may be reduced when gelatin is functionalized. In some instances, this may be desired, but in other instances, a desired functionality may be accompanied by an undesired gel strength change and/or an undesired change of the gelling temperature. Further, the gelling time is not always easily controllable, whereas in processing food or non-food products, it is desirable to control the process and gelling time. In some instances, collagens are cross-linked. In such instances, gel formation, if any, is not reversible. However, reversibility of the gelling may be desired.

Hence, it is an aspect of the invention to provide an alternative collagen, especially an alternative gelatin, which preferably further at least partly obviate one or more of above-described drawbacks. It is further an aspect of the invention to provide an alternative process for producing a gel-based product, wherein gelling can be well controlled.

In a first aspect, the invention provides a method ("method") for the production of a gel-based product ("product"), the method comprising (i) providing a liquid composition comprising a collagen, especially gelatin, especially in an amount of 0.1-30 wt.%, such as especially 0.1-10 wt.%, and (ii) adding a multivalent cation salt, especially a divalent cation salt, especially a calcium salt, to the liquid composition, especially to provide a multivalent cation concentration (especially calcium concentration) in the liquid composition up to 150 mM, such as up to 100 mM, especially up to 50 mM, wherein when combining the collagen, such as gelatin, and (calcium) salt in the liquid composition, in a specific embodiment substantially does not comprise a cross-linker, and wherein the collagen, especially gelatin, comprises amino acids with especially at least 1% of the total amount of amino acids being succinylated, phtalated or deamidated (i.e. having a degree of substitution of at least 1%). Hence, especially at least 1% of the total amount of amino acids may be functionalized with carboxylic acids. In yet a further aspect, the invention also provides a gel-based product obtainable by the method as described herein. Especially, the invention provides a gel-based product comprising 0.1-30 wt.%, such as especially 0.1-10 wt.% gelatin, comprising a multivalent cation,

especially calcium, in an amount up to 2 wt.%, the gel-based product comprising a gel phase which substantially does not comprises a cross-linker. The gel phase especially comprises said multivalent cation, such as calcium, especially in an amount up to 2 wt.% relative to the gel phase.

Especially, succinylation, phtalation or deamidation may be applied to gelatin type A. Further, especially succinylation or phtalation may be applied to gelatin type B. The phrases "succinylation, phtalation or deamidation" or "succinylation or phtalation" or similar phrases may especially indicate that one or more amino acids may be succinylated, or one or more amino acids may be phtalated, or - where applicable - one or more amino acids may be deamidated. However, these phrases or similar phrases may also indicate that one or more amino acids may be succinylated, and one or more other amino acids may be phtalated, and - where applicable – yet one or more other amino acids may be deamidated. Hence, combinations of two (or more) of these functionalizations may also be applied. Therefore, especially the invention is direct to the succinylation or phtalation of gelatin A or B, or combinations of these functionalizations and/or combinations of these gelatins.

It surprisingly appears that in such method the gel can be formed very quickly and at the moment desired during processing. Non-functionalized collagen, such as gelatin, needs substantially more time to gel, and gel formation by enzymatic cross-linking as mentioned in the prior art also takes substantially more time, even hours. It also appears that by using the specific functionalized collagen, such as gelatin, a gel is formed with good gelling properties and with properties that may be comparable to the non-functionalized collagen (non-functionalized gelatin). In other words, with functionalizing the gelling properties may be changed, or even worsened, whereas with adding calcium, or another multivalent cation, surprisingly the gel properties are restored, especially when the functionalization is chosen under the conditions as described herein. It further appears that gel formation is reversible. An advantage of especially gelatin may be that it is a molecule wherein the negative and positive charges are relative remote from each other, unlike e.g. in fibrinogen or globular proteins. Hence, with especially the choice of gelatin, unexpected processing and (gel-based) product properties are obtained. Therefore, the invention provides amongst others calcium sensitive gelatin gels or other multivalent cation gelatin gels, and more in general multivalent cation collagen gels.

It advantageously appears that (addition of) a calcium salt (or other multi-valent cation salt) can be used to initiate gelling of gelatin in a liquid composition

comprising the gelatin (which gelatin is functionalized with carboxylate groups by one or more of succinylation, phtalation and deamidation, especially succinylation), while not substantially changing one or more of the temperature and pH (though optionally also one or more these parameters may be used to induce and/or control gelling). Gelling can now be controlled much better. The gel can be formed within seconds (upon addition of the salt) at the moment desired by the process controller. Even at low temperatures, gel formation can occur within seconds. Hence, one may also use a calcium salt and gelatin in a liquid composition comprising the gelatin, wherein the gelatin is functionalized with carboxylate groups by one or more of succinylation or deamidation, to provide a reversible gel. Hence, the gelation time can be reduced considerably. Instead of succinylation, also other routes to introduce the carboxylate groups may be used. For instance, other dicarboxylic acids may be applied, like phtalic acid. Hence in yet another embodiment the gelatin is functionalized with carboxylate groups by one or more of succinylation, phtalation, etc.. Hence, e.g. one or more of succinic anhydride and phthalic anhydride, or another anhydride, may be applied to introduce the carboxylate group(s).

Here below, the invention is especially described with respect to gelatin as collagen derived material and with respect to calcium as multivalent cation. However, other multivalent cations may be relevant, such as magnesium, iron, aluminum, copper, cobalt, etc., respectively. Of course combinations of two or more different types of collagens, such as different types of gelatins, may be applied. Hence, the terms “collagen” or “gelatin”, and similar terms, may also refer to a combination of different types of collagen or different types of gelatin, respectively. Additionally or alternatively, also two or more different types of multivalent cations may be applied. Hence, the term “multivalent cation” or “calcium salt”, and similar terms, may also refer to a combination of different types of multivalent cations or different types of calcium salts, respectively.

As indicated above, the gelatin is especially functionalized with carboxylate groups. Good results are obtained when at least 10% of all lysines (of the collagen, especially gelatin) are succinylated (and/or phtalated). Succinylation is known in the art. It is a posttranslational modification where a succinyl group is added to a lysine residue in e.g. gelatin or another protein molecule. The addition of the succinyl group changes lysine's charge from +1 to −1. Hence, especially the invention also provides a method and/or product embodiment, wherein at least 10% of all lysines are succinylated (and/or phtalated), especially wherein in the range of 40-90 % of all lysines are succinylated (and/or phtalated). Instead of or in addition to succinylation (or another

process wherein the carboxylates are added, such as phthalation), also in another way negative charges may be introduced. Hence, the phrase succinylated and/or phtalated indicates that from a subset of lysines all may be phtalated, all may be succinylated, or part of the subset are phtalated and part of the subset are succinylated.

Alternatively or additionally, deamidation may be applied, to change amide groups to carboxylic acid groups. Hence, alternatively or additionally at least 2% of all asparagines and glutamines are functionalized with a carboxylic acid group, especially by deamidation. Even more especially, the invention also provides a method and/or product wherein one or more of (i) at least 50% of all asparagines and (ii) at least 15% of all glutamines are functionalized with carboxylic acid groups, apply. The phrase "wherein one or more of (i) at least 50% of all asparagines and (ii) at least 15% of all glutamines are functionalized with carboxylic acid groups, apply" and similar phrases indicate that at least 50% of all asparagines may be functionalized with carboxylic acid groups but alternatively or additionally also at least 15% of all glutamines are functionalized with carboxylic acid groups. In general, when deamidation is executed, both asparagines and glutamines are functionalized. Especially, (only) succinylation may be applied. In yet another embodiment, (only) phtalation may be applied. In yet another embodiment, FOS (fructooligosaccharides) conjugation or other functionalization may (also) be used to functionalize gelatin and control its gelling properties.

The liquid composition will in general be an aqueous liquid composition, such as water. Further, the liquid composition may comprise other components (in addition to gelatin) than the pure liquid (or liquid phase). For instance, the liquid composition may comprise further ingredients to form a food product or the liquid composition may comprise further ingredients to form an encapsulate. Hence, the liquid composition may be a viscous liquid composition. Further, the liquid composition may thus comprise in addition to a liquid phase, such as water, a plurality of components, including the collagen, especially gelatin. One or more components, such especially the collagen, even more especially gelatin, may at least partly be solved in the liquid composition. However, one or more components may also not be solved. Especially, the liquid composition may comprise a collagen, especially gelatin, especially in an amount of 0.1-30 wt.%, such as especially 0.1-10 wt.%, even more especially at least 0.5 wt.% of collagen, especially gelatin. Lower amounts may not lead to efficient formation of a gel, and at higher concentrations very viscous solutions may be obtained at high temperatures, which are difficult to handle. In general, the liquid composition may comprise the

collagen, especially gelatin, in an amount of not more than 6 wt.%. The thus obtained gel-based product may thus also comprise in the range of 0.1-30 wt.%, such as especially 0.1-10 wt.%, of the (gelled) collagen, such as (gelled) gelatin. Especially for some non-food applications, such as pharmaceutical applications, the weight percentage may be above 10 wt.%. However, the gel-based product may also an intermediate product, with the final product thus in general comprising a lower amount of (gelled) collagen than the intermediate product.

Further, it appears that with relative low amounts of the multi-valent cation salt already good gels may be obtained. Herein, multi-valent cations are cations having a charge of 2+ or higher, such as especially Ca^{2+}, which is a divalent cation. Especially calcium salts may be applied, such as calcium nitrate or calcium chloride. For non-food applications, also other salts may be applied like e.g. salts of one or more of magnesium, iron, aluminum, copper, and cobalt. Hence, in a specific embodiment (of the method) the liquid composition comprises the collagen, especially gelatin, in an amount of 1-2 wt.%, wherein the multivalent cation salt, especially calcium salt, is added to the liquid composition to provide a multivalent cation, such as calcium, concentration in the liquid composition up to 50 mM, especially up to 20 mM, and wherein the multivalent cation salt, such as calcium salt, comprises especially one or more of calcium chloride and calcium nitrate. The lower concentration of the multivalent cation salt, especially calcium, will especially be at least 0.5 mM, especially at least 5 mM.

Herein, the term cross-linking indicates a chemical bonding, especially via an organic molecule. In the present invention, due to the presence of the multivalent cation(s), collagen molecules are connected to each other via the multivalent cation(s), i.e. via ionic bonding. Hence, no cross-linker is necessary. In contrast, the presence of cross-linkers is undesired, as it leads per definition to a gel that is not reversibly formed.

As indicated above, the liquid composition in a specific embodiment thus substantially does not comprise a cross-linker. Such cross-linker may be an enzymatic cross-linker or a chemical cross-linker, as known in the art. Examples of some common cross-linkers are the imidoester cross-linker dimethyl suberimidate, the N-hydroxysuccinimide-ester cross-linker BS3, glutaraldehyde and formaldehyde. In general, the amount of cross-linker (used in the method as described herein) is smaller than 1000 ppm (mg/kg), especially smaller than 500 ppm, such as smaller than 200 ppm, even more especially smaller than 100 ppm, such as smaller than 50 ppm, especially smaller than 10 ppm, relative to the total amount of the liquid composition (or the gel-based product).

Even more especially, the amount of cross-linker (used in the method as described herein) is smaller than 1000 ppm (mg/kg), especially smaller than 500 ppm, such as smaller than 200 ppm, even more especially smaller than 100 ppm, such as smaller than 50 ppm, especially smaller than 10 ppm, relative to the total amount of the gelatin (or the gel-based product (see also below)). The enzymatic cross-linker thus especially comprises an enzyme that can induce cross-linking of proteins, like transglutaminase. Especially, when the cross-linker comprises an enzymatic cross-linker, the concentration of the enzymatic cross-linker is especially smaller than 1 enzyme units (U/ml) (at room temperature and at the pH used), such as smaller than 0.1 enzyme units, even more especially smaller than 0.01 enzyme units. Would an aldehyde-based cross-linker not be available, the amount of cross-linker can be even below 100 ppm, such as especially below 50 ppm, such as below 10 ppm, like equal to or below 1 ppm. Hence, the gel-based product as described herein may be a product wherein the amount of cross-linker is smaller than 1000 ppm (mg/kg), especially smaller than 500 ppm, especially smaller than 200 ppm, even more especially smaller than 100 ppm, such as smaller than 50 ppm, especially smaller than 10 ppm. Especially, no or less than 1000 ppm (mg/kg) (in total), especially smaller than 500 ppm, of one or more of the following components is available in the liquid composition or the gel phase in the gel-based product, respectively: glucose, a sugar, monosaccharide, a disaccharide, an aldehyde (such as glutaraldehyde, formaldehyde, glyceraldehyde), a peroxide (such as hydrogenperoxide), an epoxide (such as 1,3-butadiene diepoxide), benzene, sulfonic acid, guanidine hydrochloride, a block-aldehyde, n-methylol, a ketone, a carboxylic derivative, a carbonic acid derivate, a sulfonic ester, a sulfonyl halide, an active halogen compound, an s-triazine, an aziridine, an active oleofin, a block active oleofin, an isocyanate, a carbodiimide, an isoxazolium salt, a polymer of any one of the aforementioned monomors, a polymer containing any one of the aforementioned monomers, starch, a polysaccharide, and one or more of the above-mentioned cross-linkers. Further, especially the liquid composition or the gel-based product (especially in the gel phase thereof), the amount of cross-linking microcomponents, such as one or more of a sugar, an aldehyde, etc., may be lower than 500 ppm (mg/kg), relative to the liquid composition (or the gel phase in the gel-based product), especially in the case of gelatin type A. Optionally, the composition and/or the gel-phase of the gel-based product may include a cross-linking inhibitor. Hence, the present invention is especially related to the succinylation and/or phtalation and the deliberate addition of a divalent cation containing salt. The presence of cross-linkers may lead to less desired hardening of the gel.

The process conditions may be chosen by the person skilled in the art. In a specific embodiment, when combining the gelatin and calcium salt the liquid composition has a pH in the range 2.5-9. Further, in a specific embodiment, when combining the gelatin and calcium salt the liquid composition a temperature is applied which is especially above the melting temperature of the gelatin, but in general not above temperatures of about 70 °C. Hence, characteristic temperatures may be in the range of 30-70 °C, such as 40-70 °C, especially such as 45-50 °C.

In het method described above, the collagen may already be functionalized. However, in another embodiment, the functionalization is also included in the method as described herein. Hence, in a further embodiment, the invention also provides a method comprising (ia) providing a collagen, especially gelatin, (ib) functionalizing the collagen, especially gelatin, by one or more of succinylation (or other carboxylation) and deamidation (to provide the herein described (functionalized) collagen, especially gelatin), (ic) providing said liquid composition comprising collagen, especially gelatin, in an amount of especially 0.1-30 wt.%, such as especially 0.1-10 wt.%, and (ii) adding said multivalent cation salt, especially calcium salt, to the liquid composition to provide said multivalent cation, especially said calcium, concentration in the liquid composition especially up to 150 mM, such as up to 100 mM, especially up to 50 mM.

The gel obtained by adding the multivalent cation may have a good strength and other desirable properties (such as especially elasticity and brittleness). Especially, the thus obtained gel has a dissociation constant (or binding constant or binding affinity) of 30 mM or lower. Would one add a multivalent cation to non-functionalized gelatin, the dissociation constant would be much higher, which is indicative of a week gel. On the other hand, would a calcium sensitive enzyme be used for cross-linking, then the calcium content would have to be much higher than indicated herein values, such as at least 5 to 10 times higher than the preferred calcium contents as described herein. For non-calcium multi-valent cations the same may apply.

Different types of gelatin may be applied. As can be learned from the Gelatin Handbook (2012) of the GMIA (Gelatin Manufacturers Institute of America Gelatin) gelatin is often defined as a product obtained by the partial hydrolysis of collagen derived from the skin, white connective tissue and bones of animals. Gelatin derived from an acid-treated precursor is known as Type A and gelatin derived from an alkali-treated process is known as Type B. In the Food Chemicals Codex gelatin is defined as the product obtained from the acid, alkaline, or enzymatic hydrolysis of

collagen, the chief protein component of the of the skin, bones, and connective tissue of animals, including fish and poultry. Gelatin is thus derived from collagen which is the principal constituent of connective tissues and bones of vertebrate animals. Collagen is distinctive in that it contains an unusually high level of the cyclic amino acids proline and hydroxyproline. Collagen consists of three helical polypeptide chains wound around each other and connected by intermolecular crosslinks. Gelatin is recovered from collagen by hydrolysis. There are several varieties of gelatin, the composition of which depends on the source of collagen and the hydrolytic treatment used (see also below). The principal raw materials used in gelatin production are cattle bones, cattle hides, and pork skins. Several alternative sources include poultry and fish. Extraneous substances, such as minerals (in the case of bone), fats and albuminoids (found in skin), are removed by chemical and physical treatment to give purified collagen. These pretreated materials are then hydrolyzed to gelatin which is soluble in hot water. Gelatin may for instance include about 1-7 wt.% lysine. In a specific embodiment the gelatin comprises gelatin type A (or gelatin type B) having a Bloom in the range of 50-300. In an embodiment, the term "gelatin" may also refer to recombinant gelatin. Especially, such gelatin may now relatively easily gel due to the functionalization.

The method as described herein may be a method further including the presence of one or more other components, to provide a food product. Specific food examples wherein the thus obtained gel may be applied may e.g. be in confections such as such as gummy bears, fruit snacks, and jelly babies as well as other products such as marshmallows, gelatin desserts, ice creams, trifles, aspic, dips and yogurts. Gelatin may be used as a stabilizer, thickener, or texturizer in foods such as yogurt, cream cheese, and margarine.

Alternatively, in a specific embodiment the method as described herein may be method further including the presence of one or more other components, to provide a coating or encapsulate for a medicament. Today, gelatin is a vital ingredient in the most popular drug delivery systems in the world such as two piece hard capsules, soft capsules, tablets, coated tablets, mini, micro capsules etc. For instance, gelatin may be applied in two-piece hard capsules. The manufacture of hard gelatin capsules involves the dipping of stainless steel mold pins into the gelatin solution, drying, stripping from the pin into a collate, trimming of the caps and bodies, and joining them together. The strength, flexibility, clarity and viscous nature of gelatin provide characteristics that make it unique in the manufacture of capsules. A typical hard capsules grade gelatin

specification will have a gel strength (bloom) 200 – 270, though lower may also be possible, and may have a viscosity (mps) of 40 – 50. However, for instance, gelatin may also be applied in soft gelatin capsules. Soft gels, as they are commonly known today, use a gelatin solution that is plasticized with propylene glycol, sorbitol, glycerin or other approved mixtures. Soft gelatin capsules are one-piece and hermetically sealed to enclose a liquid or semi-liquid fill. Soft gelatin capsules are manufacture-formed, filled and sealed in one continuous operation. A typical soft capsule grade gelatin specification will have a gel Strength (bloom) 150 - 180 and may have a viscosity (mps) of 30 – 40. Gelatin may also be used in the preparation of the active ingredient and as binder in the tablet. For instance, gelatin may be used as a film former in tablet coating. Tablets are generally coated to reduce dusting, mask unpleasant taste and enable printing and color coating for product identification. Other pharmaceutical applications of gelatin include suppositories, micro-encapsulation, surgical sponges, bacterial growth media etc.

These (one or more) further ingredients, for a food product or coating or encapsulate, may be provided to the liquid composition before gelling or during gelling, or may be added to the gel-based product obtainable with the herein described method. Of course, a combination wherein during two or more stages further components (ingredients) are added may also be applied.

Hence, the method may include combining the liquid comprising gelatin and one or more other components to provide the food product or non-food product and adding the salt to induce gelling. Optionally, before combining the salt may be added, and the thereby obtained gel may be combined with the one or more other components to provide the food product. Especially, the method may include transporting the liquid comprising the gelatin to a vessel wherein or conduit wherein the liquid and the one or more other components to provide the (non-food) food product are combined, and then (also) combining the liquid comprising the gelatin with the salt, to induce gel formation. With the present method, gelling can be started at the desired time, such as within 5 minutes before combining the liquid comprising the gelatin and the one or more other components to provide the (non-food) food product, during the combining stage or after combining the liquid comprising gelatin and one or more other components to provide the food product or non-food product. This provides a better controlled processing. In this way, a food product or non-food product comprising a gel phase may be obtained. Depending upon the type of product, the gel-phase may be substantially continuous or may discontinuous (e.g. gel particles).

As indicated above, the invention also provides a gel-based product obtainable by the method as described herein. Hence, in a further aspect the invention provides (such) gel-based product, wherein the gel-based product is a food product. Hence, in yet a further aspect the invention provides (such) gel-based product, wherein the gel-based product is a coating or an encapsulate for a medicament. The term "gel-based product" indicates that the product includes a gel, even as minor component, or has been made in a process wherein a gel has been formed. Examples of such products are the above mentioned food products and non-food products. Other non-food applications of (gelled) collagens, especially (gelled) gelatin are photography, and cosmetic manufacturing. In the overview below, some possible applications are indicated.

The gel-based product may be a gel per se or may be a product including other components. For instance, the gel-based product may be a food product or a non-food product. In an embodiment, the gel may be prepared in the presence of one or more other components, to provide such food product or non-food product (or an intermediate product for a final food product or non-food product). However, in another embodiment the gel may be combined with the one or more other components to provide such food product or non-food product (or an intermediate product for a final food product or non-food product).

A preferred degree of functionalization is max 10-80%, more preferred 20-60%, most preferred 30-50%. This may apply to both the type A and B gelatin, especially when functionalized with succinate or phatalate. A preferred degree of calcium concentration (for gelling) is max 2.5-30 mM, more preferred 5-20 mM, most preferred 7.5-15 mM. This may apply to both the type A and B gelatin, especially when functionalized with succinate or phatalate.

Gelatin type A (especially succinylated) application can e.g. be a pharmaceutical soft/hard capsule production. The capsule production method may e.g. be based on (fast) gelation of the gelatin. It appears that with the gelatin functionalized as described herein, capsule production can be better controlled. Undesired cross-linking can be prevented. Gelatin type B (especially succinylated) application can e.g. be used in microencapsulation, in particular those applications were cross-linking is not desired and gelling is essential in relation to production. However, in principle both gelatin types may be used for both applications. As shown with gelatin type A or B application, instead of (or in addition to) succinylation, also phtalation may be applied. Characteristic molecular weights of gelatins that can be used in the invention may especially be in the range of

about 50-250 kDa. Further, characteristic Bloom values are in the range of 50-300, especially 80-300. Further applications of the gelatin as described herein may be in low fat spreads (e.g. mouth feel and/or stabilization of emulsion), in meat and fish (e.g. for texture and/or gelling), desserts/dairy (e.g. for texture, thickening, gelling), confectionary (e.g. for gelling, texture, chewability, stabilization, binding, etc.). Other applications include applications in juice or wine fining.

In further aspects, the invention provides a collagen-comprising product comprising a gelled collagen compound, wherein the collagen compound comprises collagen having calcium binding functionality with at least 8 per 1000 amino acids of the collagen compound, and wherein the gelled collagen compound comprises calcium bindings. Especially, the collagen compound comprises gelatin. In an embodiment the collagen compound comprises functionalized collagen, especially acylated collagen, such as succinylated collagen. In a further embodiment, the acylated collagen has a degree of acylation of at least 3 per 1000 amino acids. In a further embodiment, the collagen compound comprises deamidated collagen. Especially, the gelled collagen compound is free of enzymes having cross-linking functionality, especially wherein the gelled collagen compound is free from transglutaminase. Further, the gelled collagen compound is available in an amount of 0.3-1 wt.% relative to the total weight of the collagen-comprising product. Especially, the invention provides the use of a calcium salt and a collagen having calcium binding functionality to provide a gel. Alternatively or additionally, the invention provides the use of collagen having calcium binding functionality and a calcium salt to provide a gel with increased gelling temperature and improved gel strength. Further, the invention provides a method of making a gel, the method comprising combining collagen having calcium binding functionality with at least 8 per 1000 amino acids of the collagen compound having calcium binding functionality and a calcium source and gelling said collagen.

The term "substantially" herein, will be understood by the person skilled in the art. The term "substantially" may also include embodiments with "entirely", "completely", "all", etc. Hence, in embodiments the adjective substantially may also be removed. Where applicable, the term "substantially" may also relate to 90% or higher, such as 95% or higher, especially 99% or higher, even more especially 99.5% or higher, including 100%. The term "comprise" includes also embodiments wherein the term "comprises" means "consists of". The term "and/or" especially relates to one or more of the items mentioned before and after "and/or". For instance, a phrase "item 1 and/or item

2" and similar phrases may relate to one or more of item 1 and item 2. The term "comprising" may in an embodiment refer to "consisting of" but may in another embodiment also refer to "containing at least the defined species and optionally one or more other species". Furthermore, the terms first, second, third and the like in the description and in the claims, are used for distinguishing between similar elements and not necessarily for describing a sequential or chronological order. It is to be understood that the terms so used are interchangeable under appropriate circumstances and that the embodiments of the invention described herein are capable of operation in other sequences than described or illustrated herein.

It should be noted that the above-mentioned embodiments illustrate rather than limit the invention, and that those skilled in the art will be able to design many alternative embodiments without departing from the scope of the appended claims. In the claims, any reference signs placed between parentheses shall not be construed as limiting the claim. Use of the verb "to comprise" and its conjugations does not exclude the presence of elements or steps other than those stated in a claim. The article "a" or "an" preceding an element does not exclude the presence of a plurality of such elements. The mere fact that certain measures are recited in mutually different dependent claims does not indicate that a combination of these measures cannot be used to advantage.

The various aspects discussed in this patent can be combined in order to provide additional advantages. Furthermore, some of the features can form the basis for one or more divisional applications.

BRIEF DESCRIPTION OF THE DRAWINGS

Embodiments of the invention will now be described, by way of example only. Further, some examples will be given. Experimental results are shown in the accompanying drawings, in which:

Figure 1 shows the helicity (H) established from optical rotation (OR) measurements as described in the text for aqueous solutions of reference and succinylated gelatin with different degree of substitution (DS) as function of the temperature (T); fraction is indicated with "f"). The large squares, triangles, crosses and asterisks represent a DS of 0% (squares, upper curve between at least about 10-25 °C), 12% (diamonds; are substantially overlapped by the reference 0%), 26% (triangles) and 42% (asterisks) respectively and all collide; the materials with a DS of 65% (circles; indicated with an

arrow) and 82% (small squares (between at least about 5-35°C the lowest curve) are indicated in the figure;

Figure 2 shows the storage modulus (G') as a function of cooling/heating temperatures at 0.5°C/min scan rate for reference and gelatin solutions with different DS (3 wt %). The DS's of the different materials are indicated for the traces. The small arrows in the figure illustrate the applied temperature ramp of cooling and subsequent heating of the sample;

Figure 3 shows the calcium binding curves for 0.5 wt% solutions of (succinylated) gelatine with different DS as indicated in the graph (R = reference; 0% DS). An ensemble averaged molecular weight of 124 kDa is used to calculate B (mol bound calcium / mol protein);

Figure 4A and 4B show a qualitative assessment of 3 wt.% gelatin solutions (reference (0%) (NC refers to no Ca^{2+}) and materials with various DS) indicated on one axis at different protein concentrations (on the other axis) in terms of liquid (Aq), viscous (V), or gel (G) appearance in the absence (4A) or presence (4B) of 5 mM calcium. Protein solutions are pre-heated for 30 min at 50 °C, subsequently cooled to room temperature and assessed after 1 hour equilibration at room temperature;

Figures 5A-5B show a small deformation rheology on 3 wt.% (A) reference "R" and (B) succinylated (DS=82%) gelatin during a cooling and subsequent heating ramp between 50 and 5 °C in the presence of different calcium concentrations (0-20 mM) as indicated in the graphs;

Figure 6 shows a calorimetric analysis of the cooling ramp from 50 to 5 °C of a 3 wt% reference (left panels; R) and 82% succinylated (right panels; DS 82%) gelatin in the presence of different concentrations of calcium (Ca+ shortly indicates the Ca2+ cation. Upper panels present the onset of the gelling temperature and the lower panels show the concomitant enthalpy (E) change;

Figure 7 shows a helicity established from optical rotation (OR) measurements for reference (top panel) and 82% succinylated (lower panel) gelatin (0.5 wt %; 20 mM acetate buffer pH 6.5) in the presence of various calcium concentrations (0-20 mM) as indicated in the graphs; "f" indicates the fraction. The solid diamonds represent samples in the absence, solid squares 4mM, open triangle 10 mM and light square 20 mM of calcium; and

Figure 8 shows a uniaxial compression measurements on cylindrical self-supporting specimens (diameter of 21.7 mm and 25 mm in height) of reference gelatin

(top panel) and gelatin with a DS of 82% (lower panel) until fracture occurs in the presence of 0,4, 10 or 20 mM calcium, as indicated in the panels, with on the y-axis the true stress (TS); "R" indicates reference.

DETAILED DESCRIPTION OF THE EMBODIMENTS

The aim of the experimentation is to try to stabilize strand-strand interactions in the junction zones and thereby 'locking' the junctions. It is hypothesized that in this way the ability to take up en dissipate energy will be reduced. For that purpose gelatins are chemically modified on the available lysine-residues using succinic anhydride with varying degrees of substitution, basically converting positive charges into negative ones. Using calcium the strands, bearing now a higher negatively charge density, could become stabilized at their inter-strand junction zones. The materials are chemically and physically characterized, tested for helix propensity and thermal behavior and the impact of the modification on the mechanical behavior is studied using small and large deformation rheology.

Gelatin modification by succinylation

Gelatin type A (PGS from pork skin, 150 Bloom and approximately 124,000 Mw), kindly provided by Rousselot® (Gent, Belgium), was used for samples succinylation according to the protocol described by Kosters, H.A., Broersen, K. de Groot, J., Simons, J.W.F.A., Wierenga, P.A. and de Jongh, H.H.J. (2003), Chemical modification as a tool to generate ovalbumin variants with controlled stability, Biotechnol. Bioengineer. 84, 61-70. Samples solutions were prepared (1 wt%) by dissolving in 0.02M phosphate buffer (pH 8) and stirring at 40 °C. Preparation in water without buffer is also possible. Succinic anhydride (239690-250G, Sigma-Aldrich) were gradually and in small aliquots added (10 mg increments) to the gelatin solutions at continuous stirring up to reach the fixed amounts (5, 10, 35 mg per g of protein), in order to obtain different degrees of modification. The pH was continuously adjusted to 8.0 using a pH-stat by titration with 1M NaOH. After complete addition of the succinic anhydride and stabilization of pH, the solutions were stirred for another 30 min, followed by extensive dialysis against deionized water (at RT) and subsequently lyophilized. A reference sample was subjected to the same procedure without addition of succinic anhydride.

Chromogenic OPA Assay

To quantify the degree of succinylation (DS) in gelatin samples the availability of lysine-residues was determined using the specific reaction between ortho-phthaldialdehyde (OPA) and free primary amino groups in the proteins in the presence of 2-(dimethyl amino) ethanethiol hydrochloride (DMA), as described by Church, F.C., Swaisgood, H.E., Porter, D.H. and Catignani, G.L. (1983), Spectrophotometric assay using o-pthaldialdehyde for determination, of proteolysis in milk and isolated milk proteins, Journal of Dairy Science 66, 1219-1227. The degree of succinylation was determined by OPA (o-Phthaldialdehyde) assay adapted from Church et al.. The OPA reagent reacts with primary amino groups (N-terminus and lysine ε-amino groups) in the presence of DMA (2-(dimethyl amino) ethanethiol hydrochloride) and results in the formation of alkyl-iso-indole fluorescent moieties. The OPA reagent was prepared in a 50 ml volumetric flask by mixing 40 mg OPA dissolved in 1 ml methanol, 25 ml 0.1 M Borax solution, 200 mg DMA, 5 ml 10 wt.% SDS solution and demi water. The reagent was freshly prepared before use. The absorbance at 340 nm was measured in the spectrophotometer (UV-1800, Shimadzu) of 1 ml OPA reagent, and 1 ml OPA reagent mixed with 100 µl 0.05% succinylated SP, equilibrated for 1 h at RT in the dark. A calibration curve was obtained by measuring absorbance at 340 nm of 0.08-0.6 mM L-leucine as described above. Using this calibration line, the amount of NH_2 (mM) of protein before and after modification was obtained. Degree of modification was expressed as % of modified groups. The measured absorbance of a sample was corrected with that of a sample containing the non-reacted reagent. A calibration curve was obtained by diluting the OPA reagent with a series of L-leucine (1 mM stock) and solvent yielding concentrations of 0.66, 0.333, 0.166 and 0.083 mM. All assays were done in duplicate. DS is expressed as the percentage relative to the reference material.

Apparent isoelectric point

To determine the apparent isoelectric point, freeze dried gelatin (reference and modified), was dissolved in buffer at a concentration of 0.5 % (w/v). Zeta potential (ζ) values were determined as a function of pH at 25°C using a Zetasizer Nano ZS (Malvern Instruments, UK). Automatic titration was applied with 0.1M HNO_3 and 0.1M NaOH solutions under continuous stirring. The isoelectric point was evaluated as the pH where the detected zeta potential value of the material (mV) was zero. All experiments were performed in duplicate.

Calcium-binding measurements

A 0.1 M stock $CaCl_2$ was prepared by dissolving calcium chloride dihydrate (Sigma-Aldrich, Germany) in the appropriate acetate buffer. The concentration of free Ca^{2+} ions was measured using a calcium ion selective electrode device (Ca ISE, adapted to Orion Star A214 ISE meter). Before to sample measurements, the potentiometric readings (mV) of the electrode were calibrated using standard $CaCl_2$ solutions (0.5 to 30 mM) with addition of ISA solution to match the ionic strength in all solutions. Gelatin samples (0.9 % w/v) were titrated with the $CaCl_2$ stock-solution and the corresponding amount of mol calcium bound per mol protein (assuming an average molecular weight of 124 kDa) was established. The interaction of the potassium-acetate buffer with gelatin was neglected in the determination of calcium activity and all samples were normalized to the apparent ionic strength as described elsewhere (Determination of calcium-binding constants of caseins, phosphoserine, citrate and pyrophosphate: A modelling approach using free calcium measurement, Mekmene, O. and Gaucheron, F. (2011), Determination of calcium-binding constants of caseins, phosphoserine, citrate and pyrophosphate: A modelling approach using free calcium measurement, Food Chemistry 127, 676-682).

Calorimetric analysis

Thermodynamic effect of heating-cooling temperature ramps (60-4°C and vice versa) of succinylated solution samples 3% (w/v) in the absence or presence calcium (0, 4, 10, 20 mM concentrations) was measured by differential scanning calorimetry (TA Instrument Q1000 TzeroTM DSC). Gelatin solutions (20 µL) were placed in aluminum pans and sealed followed of heating-cooling ramps The heat flow was recorded at 2°C/min speed rate in duplicated for each modified and reference sample. Temperature and enthalpy transition values were obtained by integration of melting and cooling curves using the Universal Analysis V1.7F software (TA instruments).

Optical rotation measurements

The triple helix propensity in reference and modified gelatin - both in the absence and presence of calcium - was monitored by optical rotation (at 436nm). Thereto, a Perkin Elmer 341 Polarimeter equipped with thermostatted water bath (HAAKE Phoexix II C25P model) was used. Samples (0.5 % w/v; 20 mM acetate buffer pH 6.5) were incubated for 1 hour at 60°C in a cell with path length of 10 mm and cooled to 6 °C

by water circulation at 0.5°C/min. The specific rotation, $(\propto)\lambda=\propto/cl$, where α is the optical rotation angle in deg at λ= 436 nm, c is gelatin concentration (g/mL) and l the length of optical path cell (dm) was continuously monitored. The helix content (h) of gelatin solutions at given concentration and wavelength is determined by means of Eq (1):

$$h=((\propto)\lambda-(\propto coil)\lambda)/((\propto collagen)\lambda-(\propto coil)\lambda) \quad \text{(Eq. 1)}$$

Using the representative values for specific optical rotation (α coil)λ and (α collagen)λ native collagen (100% helicoidal) at 546 nm are -256 and -350 deg, respectively (Djabourov, M., Maquet, J., Theveneau, H., Leblond, J., and Papon, P. (1985), Kinetics of gelation of aqueous gelatin solutions, British Polymer Journal 17 (2), 169-174).

Mechanical Properties

The evolution of storage- and viscous modulus (G' and G'' respectively) were recorded with temperature ramps for modified and unmodified gelatin solutions dissolved in buffer (3% w/v) the same as different calcium addition levels. Dynamic oscillatory measurements were carried out TA Instruments AR-G2 rotational Rheometer equipped with a 4 cm concentric cylinder geometry. A slow scan rate of 0.5°C/min to permit a higher number of helical structures to be formed and stabilized junction zones was applied during cooling and heating temperature ramps from 5-50°C and inverse.

Large deformation in gelatin gel using the same solutions above mentioned but cooling down at room temperature (20 ±1°C) after 24 hours was also measured. Uniaxial compression to fracture experiments were performed using an Instron universal testing machine (model 5543, Instron International LDT, Edegem, belgium) equipped with a plate-plate geometry. A uniaxial (de)compression speed of 1 mm/s was used. Cylindrical gels were prepared pouring gelatin solutions in (previously lubricated with parafilm oil to facilitate to bring the gels out) 5-ml syringes. After to obtain self-supported gels of 10 mm (high/diameter) dimensions, samples were placed on a bottom plate to be compressed until fracture.

The determination of the recoverable energy (RE) was done by compressing the samples up to a strain of 25% at deformation speeds of 1 mm/s. The work necessary to compress the samples up to this point (W_c) was calculated from the area below the stress vs. strain curve, and recorded. After reaching 25% strain, the

samples were decompressed at the same speed and the work (W_s) released by the gel specimen was recorded. The results were expressed as RE (%) = W_s/W_c.

RESULTS

Table I present the different materials obtained when gelatin is subjected to different degrees of succinylation (DS). The DS shows a rather linear relation with the amount of succinic anhydride added, indicating that the number of lysine groups available for chemical modification is not limiting. It is also clear that, where the reference material has an IEP (iso electric point) of around 7.5, already the lowest DS yields an apparent IEP just below 5, gradually further decreasing with increasing DS. From the amino acid composition this gelatin contains about 27 lysine per 1000 residues and with an average molecular weight of 124 kDa it implies that for the highest DS about 25-27 succinate groups have been introduced per molecule. All experiments described in this work have been carried out at a pH of 6.7, this is above the isoelectric point of all succinylated variants, but below that of the reference material.

Table I Characterization of gelatin materials subjected to different degrees of succinylation (DS), as determined from the chromogenic OPA assay, and the apparent isoelectric point, as established from zeta-sizer measurements:

Material (code)	**mg succinic anhydride added per gr gelatin**	**DS (%)**	**Apparent IEP**
Reference	0	0	7.5
A	5	12	4.9
B	10	26	4.7
C	20	43	4.5
D	30	65	4.5
E	35	82	4.4

Estimated error in determination of DS as determined by the OPA-assay is ± 3%

Figure 1 shows the triple helix propensity as obtained from optical rotation measurements during a cooling ramp from 60 °C to 5 °C for aqueous solutions of reference and succinylated gelatins with different DS. It can be clearly seen that at even high DS the onset temperature for helix formation is not affected. Also, it can be observed

that up to a DS of about 60% no significant impact is observed on the helix induction, while the material with a DS of 65% is marginally affected; only for a DS of 82% a reduction of the helix propensity of about 20-25% is observed. Prolonged incubation at low temperature did not increase the helicity of this latter sample (not shown), illustrating that the observed reduction is not the result of a slower kinetic process.

Overall, it can be concluded that a series of succinylated gelatins can be prepared that, when studied above their isoelectric point, show a clear propensity to adopt a triple helical configuration essential for developing a spatial network.

Gelling behavior in the absence of calcium

The gelling properties of the various materials have been studied using small deformation rheology at concentrations well above the critical gel concentration (typically here 3 % w/v). The subsequent cooling and heating traces are shown in Figure 2. Most interestingly, the figure shows that one can eliminate almost all lysine residues and replace the positive by negative charges without losing gelling ability. It can be clearly observed that up to a DS of 65% the introduced succinate groups have only very limited impact on both gelling/melting temperatures and the gel strength developed. Only at a DS of 82% a significant loss in gel strength (factor of 5-6) was observed and the gelling temperature was shifted from 17 to 12°C. Lowering the cooling rate from 0.5 to 0.2°C/min (not shown) had only a marginal effect on the transition temperature, but no impact on G'.

The transition temperatures observed with small de formation rheology are confirmed by those obtained from thermal calorimetric analysis (Table II). Interestingly, the transition enthalpy (related to cooperative thermal processes) steadily declines with increasing DS, but shows a strong reduction for the highest DS of 82%. The energy content related to the melting process shows a much smaller dependence on the DS, illustrating that the kinetics of strand-strand assembly does play a role in network formation.

Table II Calorimetric analysis of reference and succinylated gelatin:

Material (% DS)	**Transition temperature gelling (°C)**	**Transition enthalpy gelling (J/g)**	**Transition enthalpy melting (J/g)**
Reference	20.3 ± 0.9	12.0 ± 1.8	40.2 ± 1.6

Material (% DS)	Transition temperature gelling (°C)	Transition enthalpy gelling (J/g)	Transition enthalpy melting (J/g)
12	17.8 ± 0.8	13.2 ± 0.9	39.2 ± 1.4
26	18.2 ± 0.7	9.2 ± 0.8	37.4 ± 1.6
43	17.9 ± 0.7	7.2 ± 0.5	35.9 ± 2.1
65	17.3 ± 0.6	6.3 ± 1.1	34.4 ± 1.2
82	11.8 ± 0.9	0.8 ± 0.4	28.5 ± 0.9

Calcium binding

The conceptual underlying this finding is to enable a fortification of the strand-strand interaction zones by high-affinity calcium binding. Figure 3 shows the calcium-binding curves for reference and succinylated gelatin at concentrations below the critical gel concentration (0.5% w/v). It can be observed that already the reference gelatin shows some specific calcium-binding and increasing the succinate content steadily increases the number of calcium-ions that are bound with detectable affinity to gelatin. The fact that no plateau value is reached for any of the samples indicates that the binding affinity of molecular gelatin is relatively weak and there is a continuous equilibrium between bound and non-bound calcium that is shifted towards the bound state by increasing the bulk calcium concentration.

The impact of the presence of calcium is at the physical level evaluated by assessment of the physical state of protein solutions (pre-equilibrated at 50 °C) in terms of the solutions appears liquid (free flow), viscous (a lumpy cohesive flow) or gel (immobilized) at room temperature. It can be observed in Figure 4A that for the reference gelatin a concentration higher than 0.8 % (w/v) is needed to have a viscous solution and higher than 1.0 % (w/v) to have a gel. In the absence of added calcium it can be seen that with increasing DS both the concentration to obtain a viscous or a gel state shifts to higher protein concentrations. Figure 4B shows the same samples in the same concentration range but now when cooled to room temperature in the presence of 5 mM calcium. Where for the reference gelatin no effect of added calcium can be observed, for all succinylated samples a fortification of the protein network can be observed and most effectively with higher DS. For example, gelatin with a DS of 26% behaves in the presence of 5 mM calcium physically like reference gelatin. 1% (w/v) gelatin with a DS of 82 behaves like a liquid in the absence of calcium, but shows profound viscosity in the

presence of 5 mM calcium. To elaborate on this effect, the impact of calcium on gel properties are studied for all materials in more detail. Below the gel properties of reference gelatin and that with a DS of 82% are presented only to illustrate the differences.

Calcium-sensitivity in gel formation

Figure 5 shows the small deformation rheology of 3% (w/v) gelatin solutions (reference and DS 82%) when applying a cooling ramp to pre-equilibrated samples at 50 °C to 4 °C and subsequently a heating ramp back to 50 °C in the presence of different calcium-concentrations. For reference gelatin (Figure 5A) there is no detectable impact of the presence of added calcium up to a concentration of 20 mM, nor in gelling temperature, or in the maximal gel strength developed. This holds both for the cooling and heating ramps. For the succinylated gelatin it was already shown in Figure 2 that in the absence of calcium both the gelling temperature and the gel strength developed were significantly reduced. Figure 5B shows, however, that addition of calcium 'restores' both the gelling temperature and gel strength to those observed for reference gelatin.

This behavior is confirmed when these systems were analyzed calorimetrically, as demonstrated in Figure 6. Where for reference gelatin no impact is observed of the presence of calcium both on the gelling temperature and the concomitant observed enthalpy change, for the succinylated sample the gelling transition temperature steadily increases from 11 to 17°C, where the concomitant enthalpy change increases from almost negligible in the absence of calcium to about 10 J/g in the presence of 10 mM calcium. This is still considerably lower (about 30-40%) compared to that observed for reference gelatin. In order to evaluate whether the calcium acts at the level of strand-strand interactions or at the level or in the helix assembly the optical rotation of these samples is followed during the cooling ramp. This is shown in Figure 7 where the OR is shown for reference gelatin (top panel) and for 82% succinylated gelatin (lower panel) samples at concentrations below the critical gelling concentration at different calcium concentrations. In line with the above results, calcium does not have an impact on the helix formation of reference gelatin. However, as was already shown in Figure 1, in the absence of calcium highly succinylated gelatin has a lower helix propensity. This can, as shown in the lower panel of figure 7, be partly restored by the presence of calcium. The increased helix propensity as achieved by the presence of calcium does, however, not

fully explain the enhancement of the gel mechanical properties as shown in figure 5, suggesting that the effect of calcium is not limited to the helix propensity only.

To study this in more detail the impact of 20 mM calcium on the helix propensity and maximal gel strength are presented in Table III. It can be observed in Table III that the helicity, partly restored by the addition of calcium, steadily reduces at 20 mM calcium. The maximal storage modulus on the other hand, shows a clear maximum at a DS of 43%, showing even an enhancement of the gel strength of about 20%, while the helicity is slightly reduced. At higher DS G' decreases again rather drastically, despite the fact that under these conditions the helicity is not strongly reduced.

Table III Helix propensity (optical rotation) and maximal gel strength (small deformation rheology) of reference and various succinylated gelatins in the presence of 20 mM Ca:

Material (% DS)	**Helicity (fraction) at 5 °C**	**Maximal G' (Pa)**	**Young's modulus (MPa)**
Reference	0.45 ± 0.02	760 ± 20	5.2 ± 0.5
12	0.44 ± 0.03	780 ± 30	5.3 ± 0.7
26	0.42 ± 0.02	830 ± 40	5.5 ± 0.5
43	0.40 ± 0.02	930 ± 50	5.7 ± 0.4
65	0.37 ± 0.03	550 ± 40	4.4 ± 0.6
82	0.34 ± 0.03	280 ± 30	2.3 ± 0.7

These results show that extensive succinylation of gelatin does not prohibit gel formation. Helix formation is hampered, but still profound and gel strength is only slightly reduced at very high DS. The presence of calcium restores partly the material properties to those of unmodified gelatin. Properties like helix propensity, gelling/melting temperatures and concomitant transition enthalpy changes and gel strength are shown to become calcium-sensitive. There appear to be, however, two opposing mechanisms. Increasing degree of succinylation gradually reduces the helix propensity and thereby gel rheological responses. However, succinylation increases calcium-binding with moderate affinity, leading to even significant fortification of the storage modulus at a DS of 43%. Clearly, calcium-binding does not only affect the molecular properties, but also acts as fortifier of the strand-strand interactions.

For technological impact of these results, it is most relevant to study the large deformation characteristics. The last column in Table III presents the Young's modulus as obtained from the stress-strain curve up to an applied deformation of 80% till the gel fracture point. It can be seen that also at large deformation the modulus follows that observed during small deformation. When looking at the strain needed to apply to create sufficient stress in the gel to fracture (Figure 8) it can be seen that for reference gelatin calcium again has no impact, but that the stress evoked by the applied strain leading to fracture slightly diminishes with increasing calcium concentration. This situation is very different for the gelatin with a DS of 82%. For this latter material it can be seen that in the absence of calcium only half the strain is needed, leading to also about half the stress needed to fracture the gel. Increasing the calcium-concentration leads to an almost doubling of the fracture strain, becoming even higher than that of reference gelatin.

The ability of gelatin molecules to assemble into triple helices (strands) as microstructural building block for network formation also arises from the net charge on molecules, affecting the number of junctions along a strand and their interaction efficiency. Alternatively, gelatin molecules inter-connected at random points produce shorter strands that are less flexible, produce more cross-linked or branched structures and provide consequently weaker networks. It appears that with the calcium ion, the gelling abilites of the functionalized gelatin are restored. For instance, a non-functionalized gelatin with calcium gave a brittle film, whereas gelatin functionalized as descrbed herein provides reversible gelling properties.

CLAIMS:

1. A method for the production of a gel-based product, the method comprising (i) providing a liquid composition comprising gelatin in an amount of 0.1-30 wt.%, and (ii) adding a calcium salt to the liquid composition to provide a calcium concentration in the liquid composition up to 50 mM, wherein when combining the gelatin and calcium salt the liquid composition substantially does not comprises a cross-linker, and wherein the gelatin comprises amino acids with at least 1% of the total amount of amino acids being succinylated or phtalated.

2. The method according to claim 1, wherein at least 10% of all lysines are succinylated, especially wherein in the range of 40-90 % of all lysines are succinylated.

3. The method according to claim 1, wherein at least 10% of all lysines are phtalated, especially wherein in the range of 40-90 % of all lysines are phtalated.

4. The method according to any one of the preceding claims, wherein when combining the gelatin and calcium salt the liquid composition has a pH in the range 2.5-9 of and a temperature in the range of 40-70°C.

5. The method according to any one of the preceding claims, comprising (ia) providing a gelatin, (ib) functionalizing the gelatin by one or more of succinylation and phtalation, and (ic) providing said liquid composition comprising gelatin in an amount of 0.1-10 wt.%, and (ii) adding said calcium salt to the liquid composition to provide said calcium concentration in the liquid composition up to 50 mM.

6. The method according to any one of the preceding claims, wherein the liquid composition comprises gelatin in an amount of 1-2 wt.%, wherein the calcium salt is added to the liquid composition to provide a calcium concentration in the liquid composition up to 20 mM, wherein the calcium salt comprises one or more of calcium chloride and calcium nitrate, and wherein the amount of cross-linker is smaller than 100 ppm.

7. The method according to any one of the preceding claims, wherein the gelatin comprises gelatin type A having a Bloom in the range of 50-300.

8. The method according to any one of the preceding claims, wherein the gelatin comprises gelatin type B having a Bloom in the range of 50-300.

9. The method according to any one of claims 1-8, wherein the method further includes the presence of one or more other components, to provide a food product, or a coating, or an encapsulate for a medicament.

10. A gel-based product obtainable by the method according to any one of the preceding claims.

11. A gel-based product comprising 0.1-30 wt.% gelatin, comprising calcium in an amount up to 2 wt.%, the gel-based product comprising a gel phase which substantially does not comprise a cross-linker, wherein the amount of cross-linker is smaller than 100 ppm, and wherein at least 10% of all lysines are succinylated or phtalated, and wherein the gel has a dissociation constant of 30 mM or lower.

12. The gel-based product according to any one of claims 10-11, wherein the gel-based product is a food product or wherein the gel-based product is a coating or an encapsulate for a medicament.

13. Use of a calcium salt to initiate gelling of gelatin in a liquid composition comprising the gelatin, wherein the gelatin is functionalized with carboxylate groups by one or more of succinylation or phtalation, while not substantially changing one or more of the temperature and pH.

14. Use of a calcium salt and gelatin in a liquid composition comprising the gelatin, wherein the gelatin is functionalized with carboxylate groups by one or more of succinylation or phtalation, to provide a reversible gel.

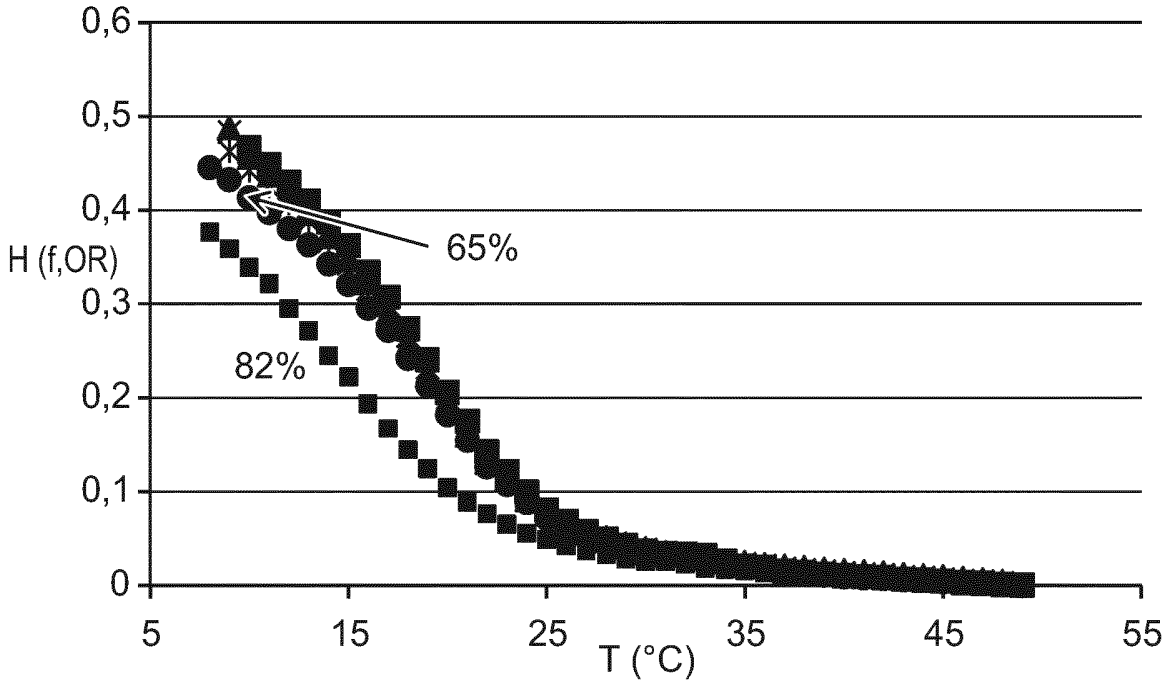
0,6
0,5
0,4
0,3
0,2
0,1
0
H (f,OR)
65%
82%
5
15
25
35
45
55
T (°C)

FIG. 1

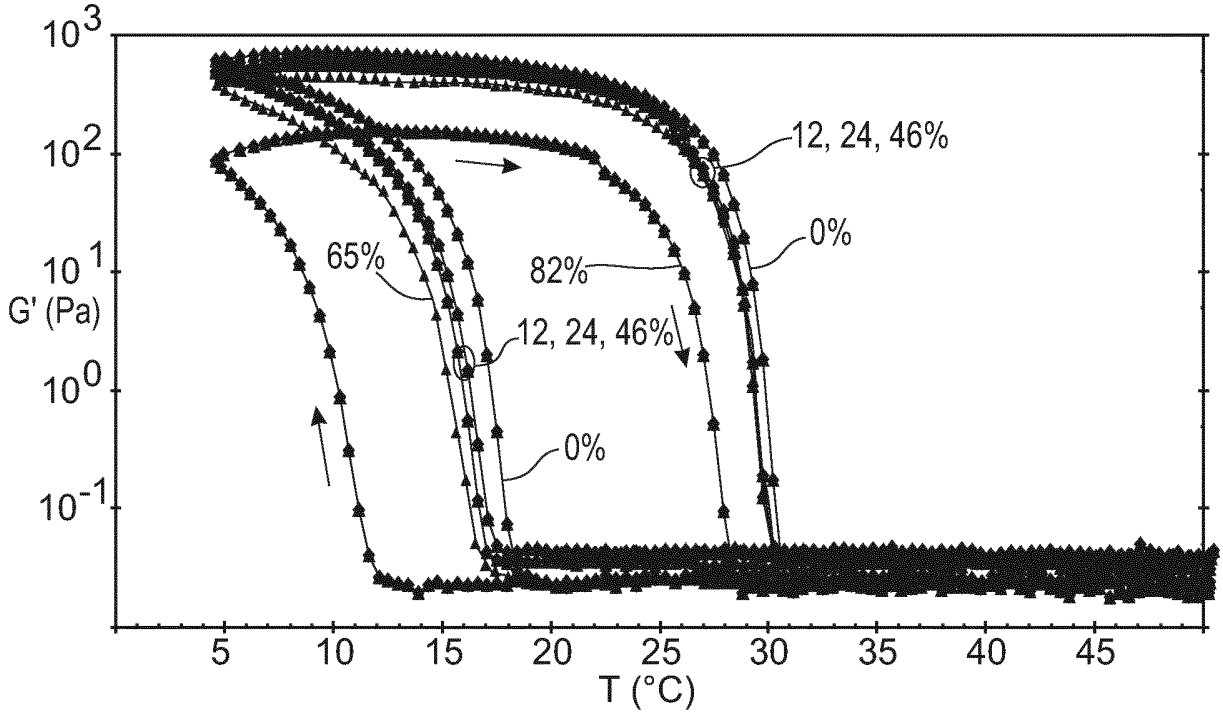
10^3
10^2
10^1
10^0
10^-1
G' (Pa)
12, 24, 46%
0%
65%
82%
12, 24, 46%
0%
5
10
15
20
25
30
35
40
45
T (°C)

FIG. 2

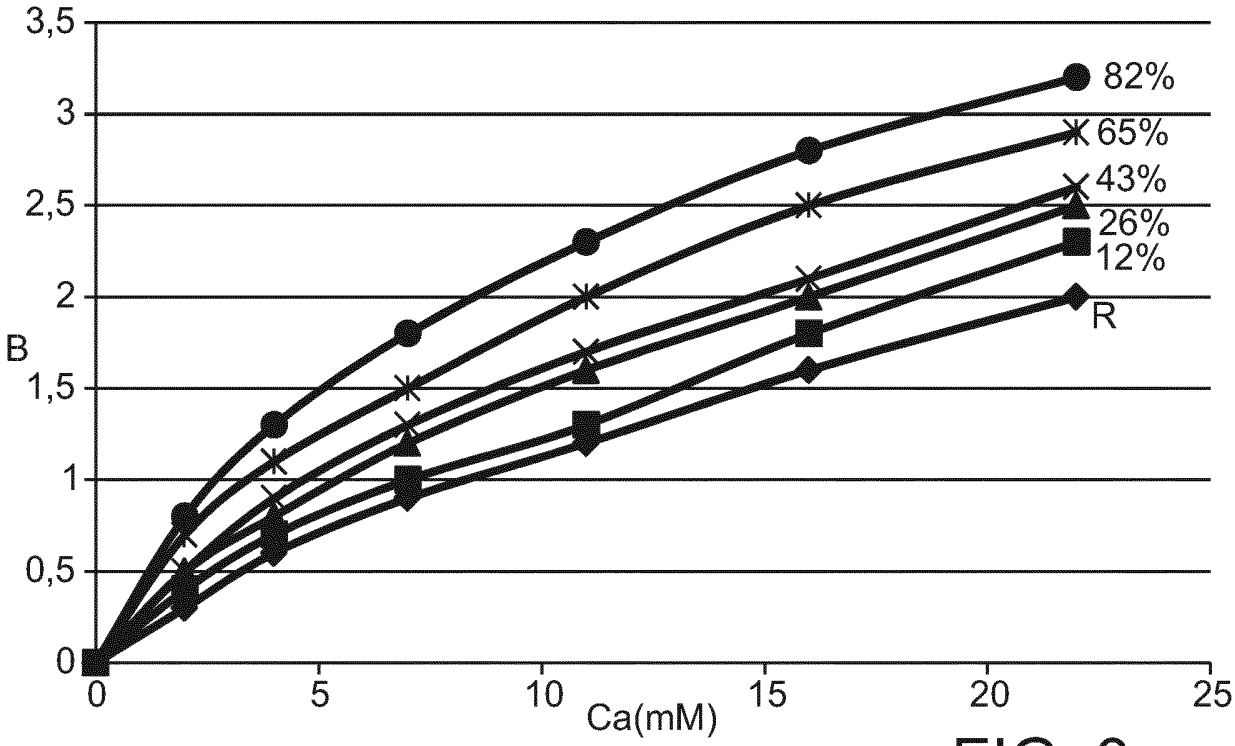

FIG. 3

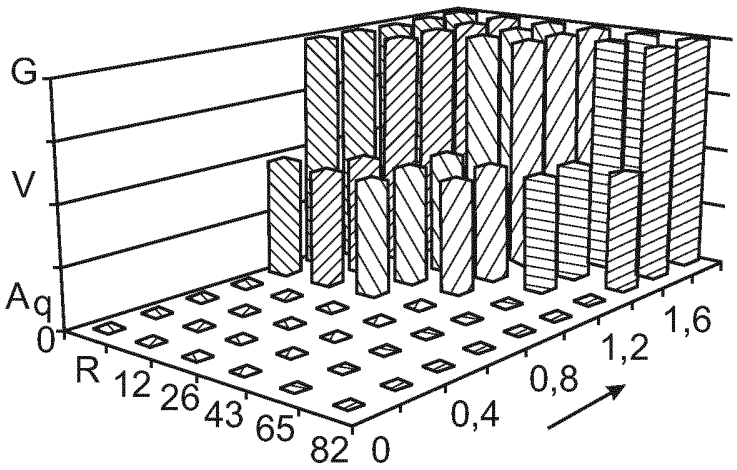

FIG. 4A

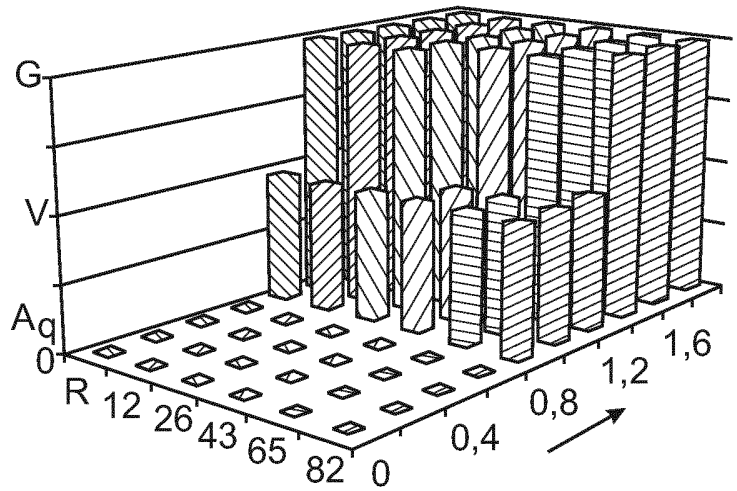

FIG. 4B

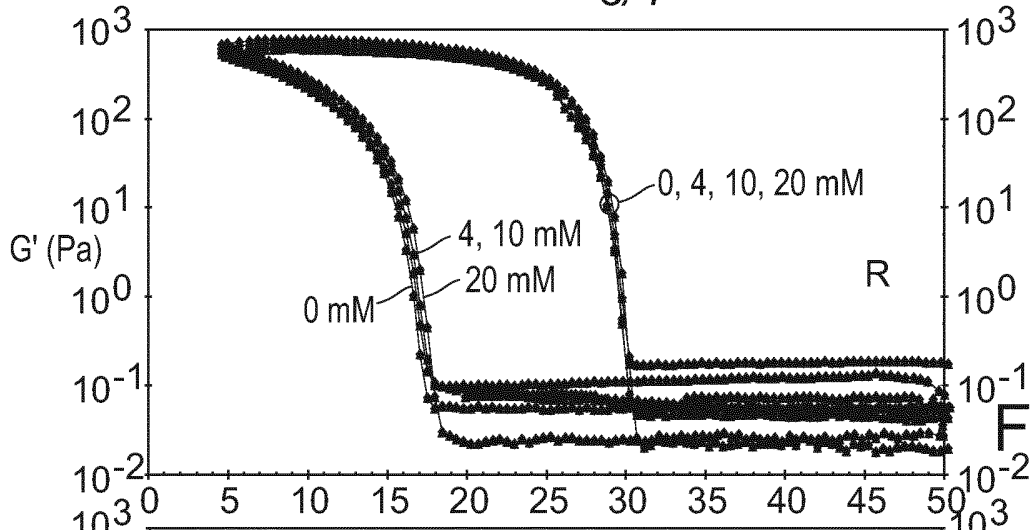

FIG. 5A

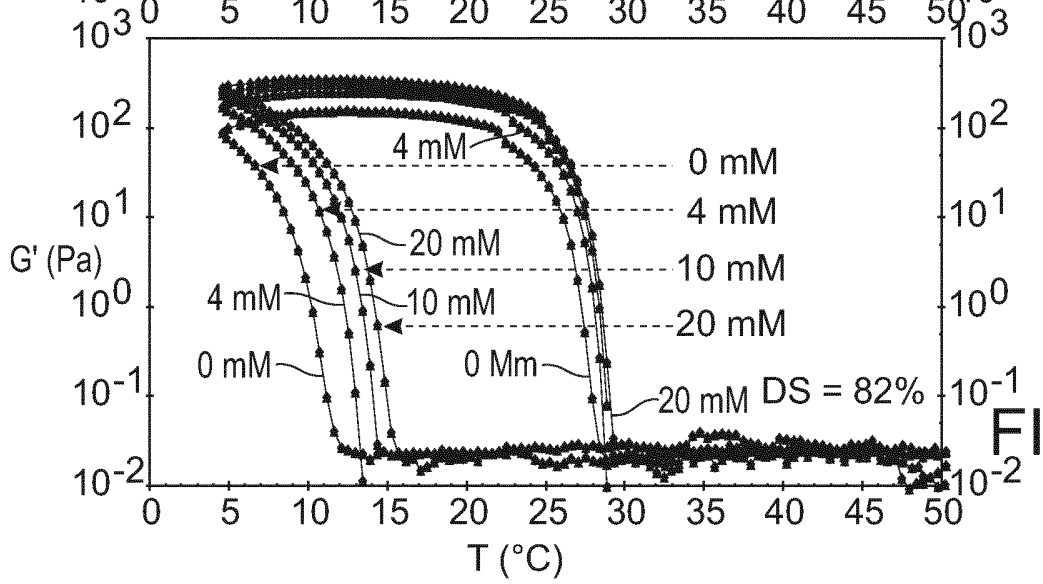

FIG. 5B

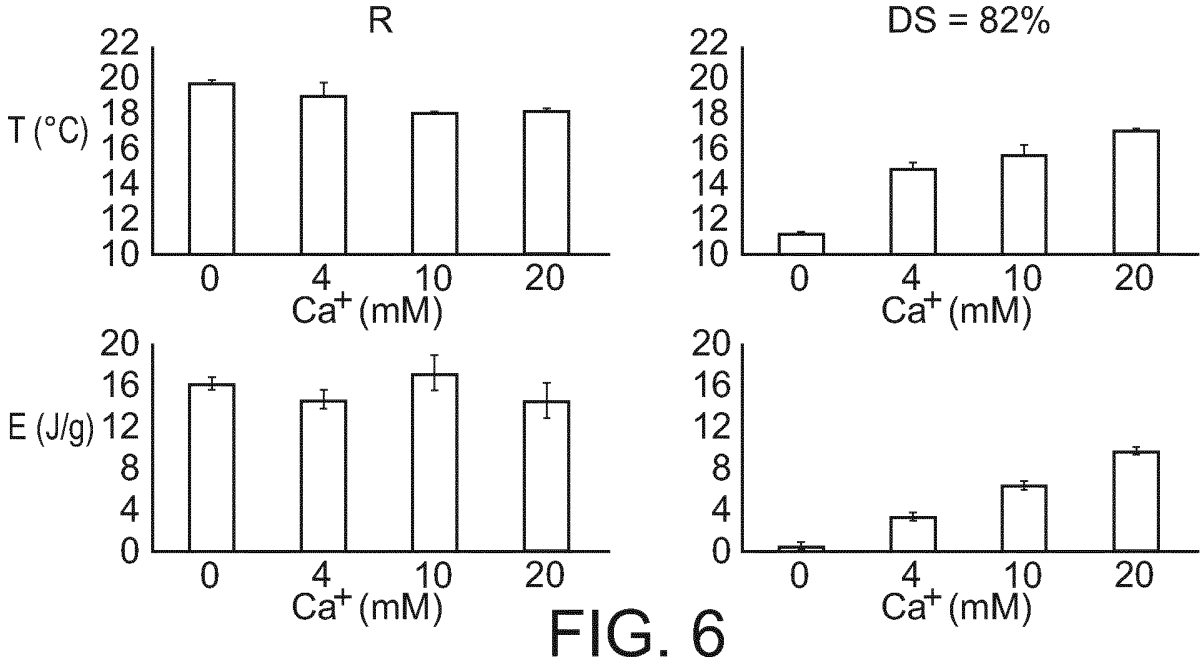

FIG. 6

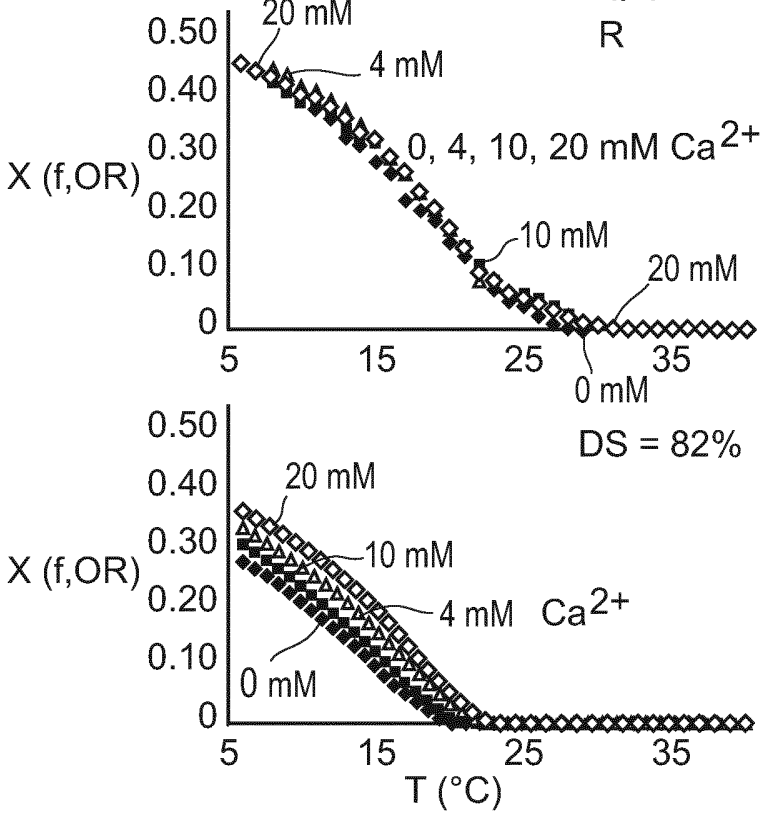
R
20 mM
4 mM
0, 4, 10, 20 mM Ca2+
10 mM
20 mM
0 mM
X (f,OR)
0.50
0.40
0.30
0.20
0.10
0
5
15
25
35
DS = 82%
20 mM
10 mM
4 mM Ca2+
0 mM
T (°C)

FIG. 7

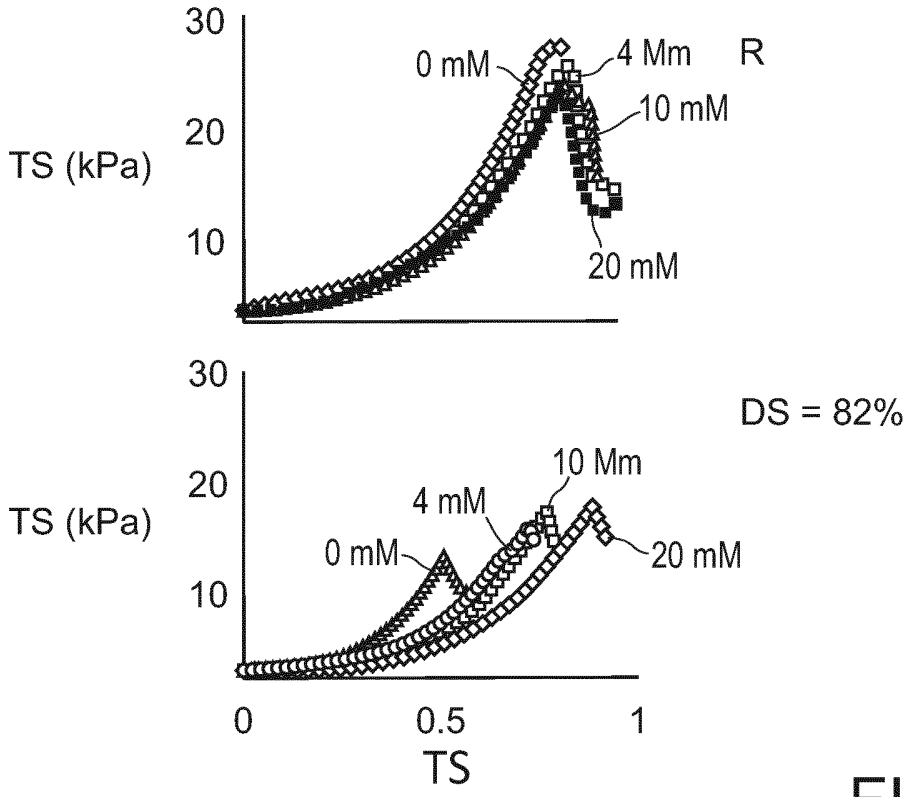
30
20
10
TS (kPa)
0 mM
4 Mm
R
10 mM
20 mM
DS = 82%
10 Mm
4 mM
0 mM
20 mM
0
0.5
1
TS

FIG. 8

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/066467

A. CLASSIFICATION OF SUBJECT MATTER
INV. A23L1/0562
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A23L

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 3 266 906 A (AARON MILLER ET AL) 16 August 1966 (1966-08-16) claim 13 -----	1-14
A	GB 1 474 891 A (INST ELEMENTOORGANICHE SOEDINE) 25 May 1977 (1977-05-25) claim 3 -----	1-14
A	EP 1 367 066 A2 (CRODA INT PLC [GB]) 3 December 2003 (2003-12-03) the whole document -----	1-14

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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

"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
14 September 2015	21/09/2015

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

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2015/066467

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 3266906 A	16-08-1966	NONE	
GB 1474891 A	25-05-1977	NONE	
EP 1367066 A2	03-12-2003	EP 1367066 A2	03-12-2003
		GB 2388776 A	26-11-2003