(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
3 October 2013 (03.10.2013)

WIPO | PCT

(10) International Publication Number
WO 2013/144247 A1

(51) **International Patent Classification**:
A61K 38/19 (2006.01) *A61P 31/04* (2006.01)
A61K 33/42 (2006.01)

(21) **International Application Number**:
PCT/EP2013/056598

(22) **International Filing Date**:
27 March 2013 (27.03.2013)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:
12161802.9 28 March 2012 (28.03.2012) EP

(71) **Applicants**: **ARLA FOODS AMBA** [DK/DK]; Sønderhøj 14, DK-8260 Viby J (DK). **AARHUS UNIVERSITET** [DK/DK]; Nordre Ringgade 1, DK-8000 Aarhus C (DK).

(72) **Inventors**: **BIRKEDAL, Henrik**; Blåbærhaven 24, DK-8520 Lystrup (DK). **OLSEN, Jakob**; Steen Billes Gade 6, 2. tv, DK-8200 Aarhus N (DK). **SKOVGAARD, Jonas**; Skanderborgvej 29, 4. th, DK-8000 Aarhus C (DK). **SCHLAFER, Sebastian**; Høegh-Guldbergsgade 123, 2. th, DK-8000 Aarhus C (DK). **MEYER, Rikke Louise**; Jeppe Aakjærs Vej 50, DK-8230 Åbyhøj (DK). **NYVAD, Bente**; Strandmarksvej 27 A, DK-8240 Risskov (DK). **SUTHERLAND, Duncan Stewart**; Klokkerfaldet 62, DK-8210 Aahus V (DK). **WEJSE, Peter Langborg**; Fridtjof Nansens Vej 25, DK-8200 Aarhus N (DK).

(74) **Agent**: **ØSTERGAARD, Steen**; Guardian IP Consulting I/S, Diplomvej, Building 381, DK-2800 Kgs. Lyngby (DK).

(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

WO 2013/144247 A1

(54) **Title**: NANOPARTICLE AGGREGATES CONTAINING OSTEOPONTIN AND CALCIUM- AND/OR STRONTIUM-CONTAINING PARTICLES

(57) **Abstract**: The present invention relates to nanoparticle aggregates comprising osteopontin (OPN) and one or more particles containing calcium and/or strontium and to their use for reducing or preventing biofilm growth or for removing biofilm. The invention furthermore relates to the use of the nanoparticle aggregates for treating, alleviating or preventing biofilm-related diseases.

Nanoparticle aggregates containing osteopontin and calcium- and/or strontium-containing particles

Technical field of the invention

The present invention relates to nanoparticle aggregates comprising osteopontin (OPN) and one or more particles containing calcium and/or strontium and to their use for reducing or preventing microbial biofilm growth or for removing microbial biofilm. The invention furthermore relates to the use of the nanoparticle aggregates for treating, alleviating or preventing biofilm-related diseases.

Background of the invention

A vast amount of damage is caused by microbial biofilms. Bacterial and fungal biofilms are involved in numerous human diseases, including bacterial endocarditis, chronic wound infections, implant infections, otitis media, caries, periodontitis and cystic fibrosis. Moreover, biofilms play an important role in food spoilage and biofouling, both of which cause huge economic losses world-wide. While conventional anti-biofilm approaches aim at the mechanical removal of biofilms and/or the killing of bacteria in the biofilms, alternative strategies target the mechanisms involved in microbial biofilm formation (adhesion, co-aggregation, biofilm maturation). Still, the harmful effects of microbial biofilms remain a major global problem. Dental caries, for example, is still the most widespread human disease.

WO2005053628 discloses the use of OPN for reducing plaque bacterial growth on tooth enamel and dental formulations containing osteopontin.

Jensen et al. (Journal of Biomedical Material Research A, Oct. 2011, Vol 99A. Issue 1) discloses hydroxyapatite nanoparticles coated with OPN and their use for implant coatings.

Holt et al. (FEBS Journal 276 (2009), pages 2308-2323) discloses the production of calcium phosphate nanoclusters using OPN or OPN fragments for controlling the growth of the calcium phosphate cores.

Summary of the invention

The inventors of the present invention have found that nanoparticle aggregates comprising OPN and a particle comprising calcium surprisingly provide a large reduce oral biofilm growth both *in vitro* and *in vivo* (see e.g. Examples 6 and 8, and the Figures 3 and 6). The nanoparticle aggregates are highly efficient in reducing biofilm formation, much more so than OPN alone, or calcium-containing particles without OPN, or other model particles. Thus, a clear synergy is obtained by combining OPN and calcium-containing particles leading to improved anti-biofilm effects. Furthermore, the inventors have shown that calcium can be replaced by strontium.

Thus, an aspect of the invention pertains to nanoparticle aggregates comprising a) OPN and b) a first particle comprising calcium and/or strontium, for use as a medicament.

Another aspect of the invention pertains to nanoparticle aggregates comprising a) OPN and b) a first particle comprising calcium and/or strontium for curing, alleviating and/or preventing a biofilm-related disease.

Yet an aspect of the invention pertains to the use of nanoparticle aggregates comprising a) OPN and b) a first particle comprising calcium and/or strontium, for reducing or preventing microbial biofilm growth or for removing microbial biofilm. In some embodiments of the invention, this use is not a treatment of the human or animal body by therapy. The biofilm may for example be a biofilm which is not in contact with a living human or animal.

An aspect of the invention relates to nanoparticle aggregates having the stoichiometric formula
$A'_{10-X}A_X(PO_4)_{6-Y}B_Y(OH)_{2-z}C_z \cdot OPN_m \cdot (H_2O)_n$; wherein A' is selected from the group consisting of Ca, Sr and mixtures thereof; wherein A is selected from the group consisting of Li, Na, K, Rb, Cs, Be, Mg, Zn, Ba, H_2O, vacancy and mixtures thereof, and X=0-9; wherein B is selected from the group consisting of (CO_3), (SO_4), (HSO_4), (HPO_4), (H_2PO_4), H_2O, vacancy and mixtures thereof, and Y=0-5; wherein C is selected from the group consisting of F, Cl, Br, I, (CO3), H_2O, vacancy and mixtures thereof, and Z=0-2; wherein n=0-10 and m>0; wherein the molar ratio between (PO_4) and (HPO_4) is above 2.5.

Another aspect of the present invention relates to the use of nanoparticle aggregates comprising a) OPN and b) strontium phosphate, calcium phosphate and mixtures of such for reducing or preventing microbial biofilm growth.

Yet another aspect of the present invention pertains to a coating composition comprising nanoparticle aggregates comprising a) OPN and b) strontium phosphate, calcium phosphate and mixtures of such.

Still another aspect of the present invention pertains to nanoparticle aggregates having the stoichiometric formula $A'_{10-X}A_X(PO_4)_{6-Y}B_Y(OH)_{2-z}C_z \cdot OPN_m \cdot (H_2O)_n$; wherein A' is selected from the group consisting of Ca, Sr and mixtures thereof; wherein A is selected from the group consisting of Li, Na, K, Rb, Cs, Be, Mg, Zn, Ba, H_2O, vacancy and mixtures thereof, and X=0-9; wherein B is selected from the group consisting of (CO_3), (SO_4), (HSO_4), (HPO_4), (H_2PO_4), H_2O, vacancy and mixtures thereof, and Y=0-5; wherein C is selected from the group consisting of F, Cl, Br, I, (CO3), H_2O, vacancy and mixtures thereof, and Z=0-2; wherein n=0-10 and m>0; wherein the molar ratio between (PO_4) and (HPO_4) is above 2.5 for use as a medicament.

Yet another aspect of the present invention pertains to nanoparticle aggregates having the stoichiometric formula $A'_{10-X}A_X(PO_4)_{6-Y}B_Y(OH)_{2-z}C_z \cdot OPN_m \cdot (H_2O)_n$; wherein A' is selected from the group consisting of Ca, Sr and mixtures thereof; wherein A is selected from the group consisting of Li, Na, K, Rb, Cs, Be, Mg, Zn, Ba, H_2O, vacancy and mixtures thereof, and X=0-9; wherein B is selected from the group consisting of (CO_3), (SO_4), (HSO_4), (HPO_4), (H_2PO_4), H_2O, vacancy and mixtures thereof, and Y=0-5; wherein C is selected from the group consisting of F, Cl, Br, I, (CO3), H_2O, vacancy and mixtures thereof, and Z=0-2; wherein n=0-10 and m>0; wherein the molar ratio between (PO_4) and (HPO_4) is above 2.5 for use as a medicament for curing, alleviating or preventing a bacterial infection.

Yet another aspect of the present invention pertains to a dental formulation comprising nanoparticle aggregates, wherein the nanoparticle aggregates have the stoichiometric formula $A'_{10-X}A_X(PO_4)_{6-Y}B_Y(OH)_{2-z}C_z \cdot OPN_m \cdot (H_2O)_n$; wherein A' is selected from the group consisting of Ca, Sr and mixtures thereof; wherein A is selected from the group consisting of Li, Na, K, Rb, Cs, Be, Mg, Zn, Ba, H_2O, vacancy and mixtures thereof, and X=0-9; wherein B is selected from the group consisting of (CO_3), (SO_4), (HSO_4), (HPO_4), (H_2PO_4), H_2O, vacancy and mixtures thereof, and Y=0-5; wherein C is selected from the group consisting of F, Cl, Br, I, (CO3), H_2O, vacancy and mixtures thereof, and Z=0-2; wherein n=0-10 and m>0; wherein the molar ratio between (PO_4) and (HPO_4) is above 2.5.

One aspect relates to a method for producing nanoparticle aggregates formed by OPN and calcium phosphate comprising:

a) Providing a first aqueous solution comprising phosphate as PO_4^{3-}; wherein the pH is within the range of 6-14;

b) Providing a second aqueous solution comprising Ca^{2+} and/or Sr^{2+}; wherein the pH is within the range of 6-14;

wherein either the first, second or both aqueous solutions comprise OPN;

c) Mixing said first and second solutions, thereby forming a suspension comprising nanoparticle aggregates comprising 1) OPN, 2) strontium phosphate, calcium phosphate or mixtures of such, and 3) water soluble electrolytes;

d) Optionally, removing a substantial amount of said water soluble electrolytes from the suspension;

e) Optionally, separating said nanoparticle aggregates from the water phase.

Brief description of the figures

Figure 1 A shows binding of OPN to bacteria in caries model biofilms. After growth phase, a biofilm was incubated with fluorescently labelled OPN for 45 min at 35 °C. Bacteria, especially chains of streptococci can be recognized, although no bacterial stain was used. The strong affinity of OPN for bacterial surfaces targets the nanoparticle aggregates towards biofilms. Bar = 10 µm. Figure 1 B shows binding of calcium phosphate nanoparticle aggregates containing OPN to *in vivo* grown dental biofilm. The biofilm was incubated with calcium phosphate nanoparticle aggregates containing OPN for 30 min at 35 °C and stained with C-SNARF-4. The calcium phosphate nanoparticle aggregates containing OPN cluster tightly around the bacterial biofilm. Bar = 20 µm;

Figure 2 shows that bacterial growth is not influenced by OPN. *S. mitis* and *A. naeslundii* were grown in THB and THB containing 0.9 g/l OPN. Bacterial growth was monitored by spectrophotometry. Error bars indicate standard deviations. OPN was shown not to have a bactericidal or bacteriostatic effect;

Figure 3 shows the effect of different agents on biofilm formation in the caries model, measured by crystal violet staining. Calcium phosphate nanoparticle aggregates containing OPN (HAP-OPN) strongly reduce the amount of biofilm formed in the flow cells, as

compared to 1000 nm polystyrene particles, silica particles (150 nm, 500 nm and 2000 nm), OPN-free calcium phosphate particle aggregates and 0.9 g/l OPN. Error bars indicate standard deviations;

Figure 4 shows that calcium phosphate nanoparticle aggregates containing OPN (HAP-OPN) bind significantly more crystal violet than silica particles, polystyrene particles, OPN in solution and OPN-free calcium phosphate nanoparticle aggregates (HAP). Crystal violet quantification of the amount of biofilm formed in the presence of nanoparticle aggregates containing OPN (shown in Figure 3) overestimates the actual amount of biofilm formed in the flow cells. Error bars indicate standard deviations;

Figure 5 shows that calcium phosphate nanoparticle aggregates containing OPN reduce the amount of biofilm grown in a caries biofilm flow cell model. Biofilms were stained with C-SNARF-4 and imaged with a confocal microscope. A: Biofilm grown without nanoparticle aggregates. B: Biofilm exposed to nanoparticle aggregates during growth. Bars = 20 μm;

Figure 6 shows that calcium phosphate nanoparticle aggregates containing OPN strongly reduce oral biofilm growth *in vivo*. A: Biofilm grown on a glass slab kept intraorally for 72 h. per day, 5-6 NaCl dips (30-60 minutes) were performed. B: Biofilm grown on a glass slab kept intraorally for 72 h. per day, 5-6 dips (30-60 min) with calcium phosphate nanoparticle aggregates containing OPN were performed. Both glass slabs were worn by the same study subject at the same time. Bars = 20 μm;

Figures 7a-7e show that calcium phosphate nanoparticle aggregates containing OPN buffer the acid produced by the strains of the caries model when grown in planktonic culture;

Figures 8a-8e show that calcium phosphate nanoparticle aggregates containing OPN buffer the acid produced by biofilms in the five-species caries model. Biofilms were only incubated with nanoparticle aggregates after growth on THB containing glucose was finished. In biofilms that were exposed to nanoparticle aggregates, pH never dropped under 5.5, the critical value for enamel dissolution;

Figure 9 shows X-ray diffraction patterns recorded using CuKα radiation. The diffraction patterns for the individual materials have been shifted vertically for clarity. Nanocrystalline apatite materials are obtained for an amount OPN added of 15 mg/ml. Above this concentration large amounts of amorphous material is observed; at 30 and 34 mg/ml very small diffraction peaks corresponding to nanocrystalline apatite are observed on top of the large amorphous background scattering;

Figure 10 shows average crystallite sizes extracted from Rietveld refinement of the X-ray diffraction data in Figure 9. The shape of nanocrystals was found to be approximately needle shaped with the long morphological axis coinciding with the crystallographic c-axis of apatite. The top panel shows results for all data while the bottom panel displays the large effect on crystallite size observed at very low concentration; the increase in crystallite size observed for 12.5 and 15 mg/ml OPN is presumably due to the formation of a mixed nanocrystalline/amorphous material;

Figure 11 shows thermogravimetric analysis (TGA) data of nanoparticle aggregates as a function of amount of OPN added. Data have been shifted along the ordinate axis for clarity. The mass loss from 25-200 °C is assigned to loss of water, while the loss from 200 to 550 °C corresponds to organic material and the loss from 550 to 1200 °C is assigned to loss of carbonate;

Figure 12 shows the mass fractions of water, organic and carbonate extracted from the TGA data in Figure 11. The organic and carbonate masses have been normalized to dry material mass (the residual mass at 200 °C);

Figure 13 shows FTIR data of nanoparticle aggregates as a function of amount of OPN added. Data have been shifted along the ordinate axis for clarity. With increasing OPN concentrations, the intensity of the amide peaks around 1300, 1550 and 1650 cm^{-1} increases, indicating more protein is associated with the particles in the high concentration synthesis. Specific peaks for phosphate (900-1200 cm^{-1}), carbonate (840-890 cm^{-1}) and amide (1595-1720 cm^{-1}) are observed; and

Figure 14 shows estimates of organic and carbonate content from IR data obtained as the ratios of peak areas for phosphate (900-1200 cm^{-1}), carbonate (840-890 cm^{-1}) and amide (1595-1720 cm^{-1}). Note the good agreement with the results obtained by TGA in Figure 12.

The present invention will now be described in more detail in the following.

Detailed description of the invention

As stated above, an aspect of the invention pertains to nanoparticle aggregates comprising a) OPN and b) a first particle comprising calcium and/or strontium, for use as a medicament.

Thus, each of the nanoparticle aggregates preferably contains both OPN and a first particle comprising calcium and/or strontium.

Calcium is preferably present in its +2 oxidation state (Ca^{2+}). Likewise, strontium is preferably used in its +2 oxidation state (Sr^{2+}).

In the context of the present invention, the phrase "Y and/or X" means "Y" or "X" or "Y and X". Along the same line of logic, the phrase "n_1, n_2, ..., n_{i-1}, and/or n_i" means " n_1" or " n_2" or ... or "n_{i-1}" or "n_i" or any combination of the components n_1, n_2,...n_{i-1}, and n_i.

As used herein the term "osteopontin" or "OPN" means osteopontin obtained from milk, including naturally occurring fragments or peptides derived from OPN by proteolytic cleavage in the milk, or genesplice-, phosphorylation-, or glycosylation variants as obtainable from the method proposed in WO 01/49741. The milk can be milk from any milk producing animals, such as cows, humans, camels, goats, sheep, dromedaries and llamas. However, OPN from bovine milk is presently preferred due to the availability. Full length osteopontin (fOPN) is an acidic, highly phosphorylated, sialic acid rich, calcium binding protein. fOPN binds 28 moles of phosphate and about 50 moles of Ca per mole. The isoelectric point of fOPN is about 3.0. The protein exists in many tissues in the body and plays a role as a signaling and regulating protein. It is an active protein in biomineralization processes. OPN is expressed by a number of cell types including bone cells, smooth muscle cells and epithelial cells.

All amounts are based on native bovine milk OPN, but can easily be corrected to the corresponding amounts of an active fraction thereof or OPN from another source. OPN or derivatives thereof can also be prepared recombinantly.

OPN is present in bovine milk, both in the form of full length bovine OPN (e.g. position 17-278 of Swiss-Prot Accession No P31096, or a peptide having at least 95% sequence identity with position 17-278 of Swiss-Prot Accession No P31096) and in the form of a long N-terminal fragment of full length bovine OPN (e.g. position 17-163 of Swiss-Prot Accession No P31096, or a peptide having at least 95% sequence identity with position 17-163 of Swiss-Prot Accession No P31096), see e.g. Bissonnette et al., Journal of Dairy Science Vol. 95 No. 2, 2012.

In the context of the present invention, the term "sequence identity" relates to a quantitative measure of the degree of identity between two amino acid sequences or between two nucleic acid sequences, preferably of equal length. If the two sequences to be

compared are not of equal length, they must be aligned to the best possible fit. The sequence identity can be calculated as

$$(N_{ref}-N_{dif})*100)/(N_{ref}),$$

wherein N_{dif} is the total number of non-identical residues in the two sequences when aligned, and wherein N_{ref} is the number of residues of the reference sequences. Hence, the DNA sequence AGTCAGTC will have a sequence identity of 75% with the sequence AATCAATC (N_{dif}=2 and N_{ref}=8). A gap is counted as non-identity of the specific residue(s), i.e. the DNA sequence AGTGTC will have a sequence identity of 75% with the DNA sequence AGTCAGTC (Ndif=2 and Nref=8). Sequence identity can for example be calculated using appropriate BLAST-programs, such as the BLASTp-algorithm provided by National Center for Biotechnology Information (NCBI), USA.

For example, the OPN used in the present invention may be substantially pure full length OPN, it may be a substantially pure fragment of full length OPN and it may be a mixture comprising full length OPN and one or more fragments of OPN.

The OPN used in the present invention may be substantially pure full length bovine OPN, it may be a substantially pure, long N-terminal fragment of full length bovine OPN, and it may be a mixture comprising full length bovine OPN and the long N-terminal fragment of full length bovine OPN. Such a mixture may for example contain full length bovine OPN in an amount of 5-40% (w/w) relative to the total amount of OPN and the long n-terminal fragment of full length bovine OPN in an amount of 60-95% (w/w) relative to the total amount of OPN.

Bovine OPN is typically available in a concentration of 20 mg OPN per litre bovine milk.

Bovine OPN can be isolated by anion exchange chromatography from e. g. acid whey at pH 4.5 as described by the patent applications WO 01/497741 A2, WO 02/28413, WO 2012/117,119 or WO 2012/117,120. An OPN purity of up to 90-95 % can be obtained.

The nanoparticle aggregates may furthermore contain other calcium binding peptides in addition to OPN.

The nanoparticle aggregates may, in addition to OPN, contain one or more phosphopeptides selected from the group consisting of fetuin A (FETUA) (Swiss-Prot Accession No P02765), proline-rich basic phosphoprotein 4 (PRB4) (Swiss-Prot Accession No P1 0163), matrix Gla protein (MGP) (Swiss-Prot Accession No P08493), secreted

phosphoprotein 24 (SPP-24) (Swiss-Prot Accession No Q13103), Riboflavin Binding Protein (Swiss-Prot Accession No P02752), integrin binding sialophosphoprotein II (IBSP-II) (Swiss-Prot Accession No P21815), matrix extracellular bone phosphoglycoprotein (MEPE) (Swiss-Prot Accession No Q9NQ76), dentin matrix acidic phosphoprotein 1 (OMP1) (Swiss-Prot Accession No Q13316), human beta-casein, bovine beta-casein, and isoforms or phophopetide fragments thereof.

Another aspect of the invention pertains to nanoparticle aggregates comprising a) OPN and b) a first particle comprising calcium and/or strontium for curing, for alleviating and/or preventing a biofilm-related disease.

The nanoparticle aggregates may be used for treating human subjects or animal subjects.

In the context of the present invention the term "biofilm-related disease" pertains to a disease which is at least partly caused by biofilm contacting the human or animal body. A biofilm-related disease may for example involve a bacterial infection.

In some preferred embodiments of the invention, the biofilm-related disease is an oral disease.

The biofilm-related disease may e.g. be dental caries, gingivitis, and/or periodontitis.

The biofilm-related disease may be gingivitis. Alternatively, the biofilm-related disease is periodontitis. The biofilm-related disease may also be dental caries.

In some embodiments of the invention, the biofilm-related disease is a disease selected from the group consisting of bacterial endocarditis, chronic wound infections, implant infections, otitis media, and cystic fibrosis, and a combination thereof.

The nanoparticle aggregates may e.g. be for curing, alleviating and/or preventing a bacterial infection, e.g. a bacterial wound infection.

An aspect of the invention may for example pertain to the nanoparticle aggregates for curing, alleviating and/or preventing a bacterial infection.

The bacterial infection may for example be an oral bacterial infection, such as gingivitis. Thus, the nanoparticle aggregates may be for curing, alleviating and/or preventing gingivitis.

The nanoparticle aggregates may be for reducing or preventing microbial biofilm growth.

For example, the nanoparticle aggregates may be for reducing or preventing the formation of dental plaque.

In addition, or alternative, to reducing or preventing microbial biofilm growth the nanoparticle aggregates may be for removing microbial biofilm, such as e.g. dental plaque.

One aspect of the present invention relates to the use of nanoparticle aggregates comprising OPN and calcium phosphate for reducing or preventing microbial biofilm growth.

A biofilm is a community of microorganisms in which cells adhere to each other on a surface. These adherent cells are frequently embedded in a self-produced matrix of extracellular polymeric substance.

Another aspect of the present invention relates to the use of nanoparticle aggregates comprising OPN and strontium/calcium phosphate for reducing or preventing microbial biofilm growth.

Still another aspect relates to the use of nanoparticle aggregates comprising OPN and strontium phosphate for reducing or preventing microbial biofilm growth.

Yet another aspect relates to the use of nanoparticle aggregates comprising OPN and mixtures of strontium phosphate and calcium phosphate for reducing or preventing microbial biofilm growth.

Another aspect relates to the use of nanoparticle aggregates comprising a) OPN and b) strontium phosphate, calcium phosphate and mixtures of such, for reducing or preventing microbial biofilm growth.

In the present context, nanoparticle aggregates are taken to mean collections of nanoparticles, wherein said nanoparticles do not readily separate from each other upon mechanical stimulus such as stirring or low-power ultrasonication. As an example, OPN nanoparticles and calcium phosphate nanoparticles combine to form nanoparticle aggregates.

Various sizes of nanoparticle aggregates may be used in the present invention. In some embodiments of the invention the nanoparticle aggregates have a hydrodynamic radius of at most 5 micron. For example, the nanoparticle aggregates may have a hydrodynamic radius of at most 2 micron. The nanoparticle aggregates may e.g. have a hydrodynamic radius of at most 1 micron. Alternatively, the nanoparticle aggregates may have a hydrodynamic radius of at most 0.7 micron.

Even smaller nanoparticle aggregates may be preferred. Thus, in some preferred embodiments of the invention the nanoparticle aggregates have a hydrodynamic radius of at most 0.5 micron. For example, the nanoparticle aggregates may have a hydrodynamic radius of at most 0.4 micron. The nanoparticle aggregates may e.g. have a hydrodynamic radius of at most 0.3 micron. Alternatively, the nanoparticle aggregates may have a hydrodynamic radius of at most 0.2 micron, such as at most 0.1 micron.

The hydrodynamic radius is preferably determined using Dynamic Light Scatter (DLS).

Typically, the nanoparticle aggregates have a hydrodynamic radius of at least 5 nm, and preferably at least 10 nm.

As said, the first particle comprises calcium and/or strontium.

In some preferred embodiments of the invention the first particle comprises calcium.

In some preferred embodiments of the invention the first particle comprises strontium.

In some preferred embodiments of the invention the first particle comprises calcium and strontium.

The first particle may e.g. comprise, or even consist of, a salt comprising calcium and/or strontium.

The first particle may comprises at least 50% (w/w) of the salt of calcium and/or strontium relative to the total weight of the first particle. For example, the first particle may comprise at least 70% (w/w) of the salt of calcium and/or strontium. The first particle may e.g. comprise at least 80% (w/w) of the salt of calcium and/or strontium. Alternatively, the first particle may comprise at least 90% (w/w) of the salt of calcium and/or strontium, such as at least 95% (w/w.)

The salt may be an organic salt containing calcium and/or strontium and an appropriate organic anion, e.g. in the form of an anionic organic polymer.

Alternatively, the salt may be an inorganic salt containing calcium and/or strontium and an appropriate inorganic anion. None-limiting examples of such inorganic anions are a phosphate species, a sulfate, a carbonate, or a mixture thereof. Thus, the inorganic salt may comprise a phosphate species, sulfate, and/or carbonate.

The phosphate species may for example be phosphate (PO_4^{3-}), monohydrogen phosphate (HPO_4^{2-}), a pyrophosphate, or diphosphate ($P_2O_7^{4-}$). It is presently preferred that the phosphate species is phosphate (PO_4^{3-}), or alternatively a combination of phosphate (PO_4^{3-}) and monohydrogen phosphate (HPO_4^{2-}).

Thus, in some preferred embodiments of the invention, the first particle comprises, or even consists of, an inorganic salt of calcium and/or strontium.

Preferably, the first particle comprises at least 50% (w/w) of inorganic salt of calcium and/or strontium relative to the total weight of the first particle. For example, the first particle may comprise at least 70% (w/w) of inorganic salt of calcium and/or strontium. The first particle may e.g. comprise at least 80% (w/w) of inorganic salt of calcium and/or strontium. Alternatively, the first particle may comprise at least 90% (w/w) of inorganic salt of calcium and/or strontium, such as at least 95% (w/w.)

In some preferred embodiments of the invention the first particle is capable of releasing calcium and/or strontium. This release of calcium and/or strontium preferably occurs when the nanoparticle aggregates contact or are near the biofilm. For example, the nanoparticle aggregates are preferably capable of releasing calcium and/or strontium when present in a liquid film of the oral cavity, such as e.g. saliva or an oral biofilm.

In some presently preferred embodiments of the invention, the first particle is a nanoparticle, i.e. it has a hydrodynamic radius of at most 1 micron. For example, the hydrodynamic radius of the first particle may be at most 0.8 micron. The hydrodynamic radius of the first particle may e.g. be at most 0.6 micron. Alternatively, the hydrodynamic radius of the first particle may be at most 0.4 micron.

Even smaller particles may be preferred, thus the first particle may have a hydrodynamic radius of at most 0.2 micron. For example, the hydrodynamic radius of the first particle may be at most 0.1 micron. The hydrodynamic radius of the first particle may e.g. be at

most 0.05 micron. Alternatively, the hydrodynamic radius of the first particle may be at most 0.01 micron.

Typically, the hydrodynamic radius of the first particle is at least 3 nm and preferably at least 5 nm.

In some preferred embodiments of the invention at least some of the nanoparticle aggregates contain a single first particle to which one or more OPN molecules are bound. The first particle of the nanoparticle aggregates may for example be surrounded by a monolayer of OPN. Examples of such nanoparticle aggregates can be found in Holt et al. (FEBS Journal; vol. 276; pages 2308-2323; 2009).

In some preferred embodiments of the invention at least some of the nanoparticle aggregates comprise a second particle, and possibly even further particles, of the same type as the first particle. Such nanoparticle aggregates are therefore more complex structures each contain multiple particles containing calcium and/or strontium in addition to multiple OPN molecules.

In some preferred embodiments of the invention, the first particle comprises, or even consists essentially of, calcium phosphate. For example, the first particle may comprise a total amount of calcium phosphate of at least 50% (w/w) relative to the weight of the first particle. The first particle may e.g. comprise a total amount of calcium phosphate of at least 60% (w/w) relative to the weight of the first particle, such as at least 70% (w/w) or even at least 80% (w/w). Alternatively, the first particle may comprise a total amount of calcium phosphate of at least 90% (w/w) relative to the weight of the first particle. The remaining part of the first particle may e.g. be impurities from the sources of calcium and phosphate used to prepare the first particle.

The first particle may e.g. have the stoichiometric formula:
$A'_{10-X}A_X(PO_4)_{6-Y}B_Y(OH)_{2-z}C_z \cdot (H_2O)_n$; wherein A' is selected from the group consisting of Ca, Sr and mixtures thereof; wherein A is selected from the group consisting of Li, Na, K, Rb, Cs, Be, Mg, Zn, Ba, H_2O, vacancy and mixtures thereof, and X=0-9; wherein B is selected from the group consisting of (CO_3), (SO_4), (HSO_4), (HPO_4), (H_2PO_4), H_2O, vacancy and mixtures thereof, and Y=0-5; wherein C is selected from the group consisting of F, Cl, Br, I, (CO3), H_2O, vacancy and mixtures thereof, and Z=0-2; and wherein n=0-10; wherein the molar ratio between (PO_4) and (HPO_4) is above 2.5.

Another aspect of the invention relates to nanoparticle aggregates having the stoichiometric formula

$A'_{10-X}A_X(PO_4)_{6-Y}B_Y(OH)_{2-Z}C_Z \cdot OPN_m \cdot (H_2O)_n$; wherein A' is selected from the group consisting of Ca, Sr and mixtures thereof; wherein A is selected from the group consisting of Li, Na, K, Rb, Cs, Be, Mg, Zn, Ba, H_2O, vacancy and mixtures thereof, and X=0-9; wherein B is selected from the group consisting of (CO_3), (SO_4), (HSO_4), (HPO_4), (H_2PO_4), H_2O, vacancy and mixtures thereof, and Y=0-5; wherein C is selected from the group consisting of F, Cl, Br, I, (CO3), H_2O, vacancy and mixtures thereof, and Z=0-2; wherein n=0-10 and m>0; wherein the molar ratio between (PO_4) and (HPO_4) is above 2.5.

In the present context, the term 'vacancy' is to be understood as a type of point defect in the mineral. Mineral crystals inherently possess imperfections, often referred to as 'crystalline defects'. A defect wherein an atom is missing from one of the lattice sites is known as a 'vacancy' defect.

In one embodiment, A' is Ca.

In another embodiment, A' is Sr.

In yet another embodiment, A' is a mixture of Ca and Sr.

In one embodiment, A is selected from the group consisting of Na, K, Rb, Cs, Mg, Zn, Ba, vacancy and mixtures thereof.

In another embodiment, A is selected from the group consisting of Na, K, Mg, Zn, Ba, vacancy and mixtures thereof.

In still another embodiment, A is selected from the group consisting of Na, K, vacancy and mixtures thereof.

In yet another embodiment, B is selected from the group consisting of (CO_3), (HPO_4), (H_2PO_4), H_2O, vacancy and mixtures thereof.

In yet another embodiment, C is selected from the group consisting of F, Cl, vacancy and mixtures thereof.

In one embodiment, the molar ratio between (PO_4) and (HPO_4) is above 2.5, such as above 5, such as above 10, such as above 15, such as above 20, such as above 25, such as above 30, such as above 35, such as above 40, such as above 45, such as above 50, such as above 100, such as above 200, such as above 300, such as above 400, such as above

500, such as above 1000, such as above 10,000, such as above 20,000, such as above 50,000, such as above 100,000.

In yet another embodiment, A' is a mixture of Ca and Sr; wherein the ratio between Ca and Sr is within the range of 1:1000 to 1000:1, such as within the range of 1:900 to 900:1, e.g. 1:850 or 850:1, such as within the range of 1:800 to 800:1, e.g. 1:750 or 750:1, such as within the range of 1:700 to 700:1, e.g. 1:650 or 650:1, such as within the range of 1:600 to 600:1, e.g. 1:550 or 550:1, such as within the range of 1:500 to 500:1, e.g. 1:450 or 450:1, such as within the range of 1:400 to 400:1, e.g. 1:350 or 350:1, such as within the range of 1:300 to 300:1, e.g. 1:250 or 250:1, such as within the range of 1:200 to 200:1, e.g. 1:150 or 150:1, such as within the range of 1:100 to 100:1, e.g. 1:75 or 75:1, such as within the range of 1:50 to 50:1, e.g. 1:25 or 25:1, such as within the range of 1:15 to 15:1, e.g. 1:10 or 10:1, such as within the range of 1:5 to 5:1, e.g. 1:5 or 5:1, such as within the range of 1:2.5 to 2.5:1, e.g. 1:2.5 or 2.5:1, e.g. 1:1.

In one embodiment, X is within the range of 0-9, such as within the range of 0-8, such as within the range of 0-7, such as within the range of 0-6, such as within the range of 0-5, such as within the range of 0-4, such as within the range of 0-3, such as within the range of 0-2, such as within the range of 0-1.

In another embodiment, X is within the range of 0-9.5, such as within the range of 0.1-9.0, e.g. 0.2, such as within the range of 0.3-8.5, e.g. 0.4, such as within the range of 0.5-8.0, e.g. 0.6, such as within the range of 0.7-7.5, e.g. 0.8, such as within the range of 0.9-7.0, e.g. 1.0, such as within the range of 1.1-6.5, e.g. 1.2, such as within the range of 1.3-6.0, e.g. 1.4, such as within the range of 1.5-5.5, e.g. 1.6, such as within the range of 1.7-5.0, e.g. 1.8, such as within the range of 1.9-4.5, e.g. 2.0, such as within the range of 2.1-4.0, e.g. 2.2, such as within the range of 2.3-3.5, e.g. 2.4, such as within the range of 2.5-3.5, e.g. 3.0.

In one embodiment, Y is within the range of 0-5, such as within the range of 0-4, such as within the range of 0-3, such as within the range of 0-2, such as within the range of 0-1.

In another embodiment, Y is within the range of 0-4.5, such as within the range of 0.1-4.0, e.g. 0.2, such as within the range of 0.3-4.0, e.g. 0.4, such as within the range of 0.4-4.0, e.g. 0.5, such as within the range of 0.6-4.0, e.g. 0.7, such as within the range of 0.8-4.0, e.g. 0.9, such as within the range of 1.0-3.5, e.g. 1.2, such as within the range of 1.3-3.5, e.g. 1.4, such as within the range of 1.5-3.5, e.g. 1.6, such as within the range of 1.7-3.5, e.g. 1.8, such as within the range of 1.9-3.5, e.g. 2.0, such as within the range of

2.1-3.0, e.g. 2.2, such as within the range of 2.3-3.0, e.g. 2.4, such as within the range of 2.5-3.0.

In one embodiment, Z is within the range of 0-2, such as within the range of 0-1.

In another embodiment, Z is within the range of 0-1.9, such as within the range of 0.1-1.9, e.g. 0.2, such as within the range of 0.3-1.9, e.g. 0.4, such as within the range of 0.5-1.9, e.g. 0.6, such as within the range of 0.7-1.9, e.g. 0.8, such as within the range of 0.9-1.9, e.g. 1.0, such as within the range of 1.0-1.8, e.g. 1.1, such as within the range of 1.2-1.7, e.g. 1.3, such as within the range of 1.4-1.6, e.g. 1.5.

In one embodiment, B is selected from the group consisting of (CO3), H_2O, vacancy and mixtures thereof.

In another embodiment, A is selected from the group consisting of Na, H_2O, vacancy and mixtures thereof.

In yet another embodiment, B is selected from the group consisting of (CO3), H_2O, vacancy and mixtures thereof; and A is selected from the group consisting of Na, H_2O, vacancy and mixtures thereof.

In yet another embodiment, B is selected from the group consisting of (CO3), H_2O, vacancy and mixtures thereof; and A is selected from the group consisting of K, H_2O, vacancy and mixtures thereof.

In one embodiment, m is within the range of $1*10^{-10}$ to 0.25, such as within the range of $1*10^{-10}$ to 0.20, e.g. within the range of $1*10^{-10}$ to 0.15, such as within the range of $1*10^{-10}$ to 0.10, e.g. within the range of $1*10^{-10}$ to 0.09, such as within the range of $1*10^{-10}$ to 0.08, e.g. within the range of $1*10^{-10}$ to 0.07, such as within the range of $1*10^{-10}$ to 0.06, e.g. within the range of $1*10^{-10}$ to 0.05, such as within the range of $1*10^{-10}$ to 0.04, e.g. within the range of $1*10^{-10}$ to 0.03, such as within the range of $1*10^{-10}$ to 0.02, e.g. within the range of $1*10^{-10}$ to 0.01.

In yet another embodiment, m is within the range of $1*10^{-10}$ to 0.30, such as within the range of $1*10^{-9}$ to 0.20, e.g. within the range of $1*10^{-8}$ to 0.15, such as within the range of $1*10^{-7}$ to 0.10, e.g. within the range of $1*10^{-6}$ to 0.09, such as within the range of $1*10^{-5}$ to 0.08, e.g. within the range of $1*10^{-4}$ to 0.07, such as within the range of 0.001 to 0.06, e.g. within the range of 0.002 to 0.05, such as within the range of 0.003 to 0.04,

e.g. within the range of 0.005 to 0.03, such as within the range of 0.006 to 0.02, e.g. within the range of 0.007 to 0.01.

In still another embodiment, m is within the range of 0.00016 to 0.011, such as 0.00016 to 0.009, such as 0.0009 to 0.0035, such as 0.0018 to 0.003.

In another embodiment, n is within the range of 0-100, such as 0.01-100, e.g. 0.05, such as within the range of 0.1-90, e.g. 0.2, such as within the range of 0.3-85, e.g. 0.4, such as within the range of 0.5-80, e.g. 0.6, such as within the range of 0.7-75, e.g. 0.8, such as within the range of 0.9-70, e.g. 1.0, such as within the range of 1.2-65, e.g. 1.4, such as within the range of 1.6-60, e.g. 1.8, such as within the range of 2.0-55, e.g. 2.2, such as within the range of 2.4-50, e.g. 2.4, such as within the range of 2.6-45, e.g. 2.8, such as within the range of 3.0-40, e.g. 3.2, such as within the range of 3.4-35, e.g. 3.6, such as within the range of 3.8-30, e.g. 4.0, such as within the range of 4.2-25, e.g. 4.4, such as within the range of 4.6-20, e.g. 4.8, such as within the range of 5.0-15, e.g. 5.2, such as within the range of 5.4-14, e.g. 5.6, such as within the range of 5.8-13, e.g. 6.0, such as within the range of 6.2-12, e.g. 6.4, such as within the range of 6.6-11, e.g. 6.8, such as within the range of 7.0-10, e.g. 7.2, such as within the range of 7.4-9, e.g. 7.6, such as within the range of 7.8-8.8, e.g. 8.0.

Still another aspect of the present invention pertains to nanoparticle aggregates having the stoichiometric formula $A'_{10-X}A_X(PO_4)_{6-Y}B_Y(OH)_{2-z}C_z \cdot OPN_m \cdot (H_2O)_n$; wherein A' is selected from the group consisting of Ca, Sr and mixtures thereof; wherein A is selected from the group consisting of Li, Na, K, Rb, Cs, Be, Mg, Zn, Ba, H_2O, vacancy and mixtures thereof, and X=0-9; wherein B is selected from the group consisting of (CO_3), (SO_4), (HSO_4), (HPO_4), (H_2PO_4), H_2O, vacancy and mixtures thereof, and Y=0-5; wherein C is selected from the group consisting of F, Cl, Br, I, (CO3), H_2O, vacancy and mixtures thereof, and Z=0-2; wherein n=0-10 and m>0; wherein the molar ratio between (PO_4) and (HPO_4) is above 2.5 for use as a medicament.

Yet another aspect of the present invention pertains to nanoparticle aggregates having the stoichiometric formula $A'_{10-X}A_X(PO_4)_{6-Y}B_Y(OH)_{2-z}C_z \cdot OPN_m \cdot (H_2O)_n$; wherein A' is selected from the group consisting of Ca, Sr and mixtures thereof; wherein A is selected from the group consisting of Li, Na, K, Rb, Cs, Be, Mg, Zn, Ba, H_2O, vacancy and mixtures thereof, and X=0-9; wherein B is selected from the group consisting of (CO_3), (SO_4), (HSO_4), (HPO_4), (H_2PO_4), H_2O, vacancy and mixtures thereof, and Y=0-5; wherein C is selected from the group consisting of F, Cl, Br, I, (CO3), H_2O, vacancy and mixtures thereof, and Z=0-2; wherein n=0-10 and m>0;

wherein the molar ratio between (PO_4) and (HPO_4) is above 2.5 for use as a medicament for curing, alleviating or preventing a bacterial infection.

In one embodiment, A is selected from the group consisting of Zn, vacancy and mixtures thereof.

In another embodiment, B is selected from the group consisting of (CO3), H_2O, vacancy and mixtures thereof.

In yet another embodiment, C is selected from the group consisting of F, H_2O, vacancy and mixtures thereof.

In yet another embodiment, A is selected from the group consisting of Na, H_2O, vacancy and mixtures thereof, and X=0-9; wherein B is (CO_3) and Y=0-5; wherein C is selected from the group consisting of H_2O, vacancy and mixtures thereof, and Z=0-2; wherein n=0-10 and m>0.

In one embodiment, A is selected from the group consisting of Na, H_2O, vacancy and mixtures thereof, and X=0-2; wherein B is (CO_3) and Y=0-1; wherein C is selected from the group consisting of H_2O, vacancy and mixtures thereof, and Z=0-2; wherein n=0-10 and m>0.

In one embodiment, A is selected from the group consisting of Na, Zn, H_2O, vacancy and mixtures thereof, and X=0-9; wherein B is selected from the group consisting of (CO_3), (HPO_4), (H_2PO_4), H_2O, vacancy and mixtures thereof, and Y=0-5; wherein C is selected from the group consisting of F, (CO3), H_2O, vacancy and mixtures thereof, and Z=0-2; wherein n=0-10 and m>0; wherein the molar ratio between (PO_4) and (HPO_4) is above 2.5.

In another embodiment, the nanoparticle aggregates are amorphous. The first particle may for example be substantially amorphous.

Alternatively, the first particle may be substantially crystalline.

In yet another embodiment, the nanoparticle aggregates contain crystalline material matching the X-ray diffraction pattern of hydroxylapatite.

In the present context, the term 'nanocrystalline' is to be understood as a crystalline material where at least one dimension of the nanocrystals is smaller than 100 nm.

In still another embodiment, the nanoparticle aggregates contain crystalline material matching the X-ray diffraction pattern of hydroxylapatite and said crystalline material is nanocrystalline.

In one embodiment, the nanoparticle aggregates contain crystalline material matching the X-ray diffraction pattern of hydroxylapatite, and said crystalline material is nanocrystalline; wherein said nanocrystals have anisotropic crystallite size with the crystallographic c-axis coinciding with the largest morphological axis of the crystallites.

In another embodiment, the nanoparticle aggregates contain crystalline material matching the X-ray diffraction pattern of hydroxylapatite and said crystalline material is nanocrystalline in the sense that at least one dimension of the nanocrystals is smaller than 90 nm, such as smaller than 80 nm, e.g. smaller than 70 nm, such as smaller than 60 nm, e.g. smaller than 50 nm, such as smaller than 40 nm, e.g. smaller than 30 nm, such as smaller than 20 nm, e.g. smaller than 10 nm.

The inventors have seen indications that the present nanoparticle aggregates are particularly suitable for preventing or destabilising biofilm which contains one or more species of OPN-binding bacteria.

In some embodiments of the invention the biofilm contains bacteria having an OPN binding capacity of at least 50 OPN molecules per cell. For example, the biofilm may contain bacteria having an OPN binding capacity of at least 100 OPN molecules per cell. The biofilm may e.g. contain bacteria having an OPN binding capacity of at least 200 OPN molecules per cell. Alternatively, the biofilm may contain bacteria having an OPN binding capacity of at least 400 OPN molecules per cell.

The OPN binding capacity of a bacterial strain is measured according to Rydén et al., Eur. J. Biochem., 184, 331-336 (1989) using full length OPN isolated from bovine milk.

In some preferred embodiments of the invention the biofilm contains bacteria having an OPN binding capacity of at least 800 OPN molecules per cell. For example, the biofilm may contain bacteria having an OPN binding capacity of at least 2,000 OPN molecules per cell. The biofilm may e.g. contain bacteria having an OPN binding capacity of at least 10,000 OPN molecules per cell. Alternatively, the biofilm may contain bacteria having an OPN binding capacity of at least 50,000 OPN molecules per cell, such as e.g. at least 100,000 OPN molecules per cell or even at least 500,000 OPN molecules per cell.

The biofilm may e.g. contain bacteria having an OPN binding capacity of at least 1,000,000 OPN molecules per cell.

For example, the biofilm may contain, or even consist of, one or more bacteria selected from the group consisting of *Streptococcus spp., Staphylococcus spp., Pseudomonas spp. Actinomyces spp., Lactobacillus spp., Aggregatibacter spp., Bacteroides spp., Listeria spp., Campylobacter spp., Eikenella spp., Porphyromonas spp., Prevotella spp., Treponema spp.,* and combinations thereof.

In the case of gingivitis, the biofilm typically contains one or more of the bacteria *Aggregatibacter actinomycetemcomitans, Bacteroides forsythus, Campylobacter rectus, Eikenella corrodens*, *Porphyromonas gingivalis, Prevotella intermedia, Prevotella nigrescens*, and/or *Treponema denticola.*

In the case of dental caries the biofilm typically contains one or more of the bacteria *Streptococcus oralis*, *Streptococcus downei*, *Streptococcus mitis*, *Streptococcus sanguinis* and *Actinomyces naeslundii*.

The biofilm may furthermore, in addition to the OPN-binding bacteria, contain bacteria having no or low binding to OPN.

One aspect relates to the use of nanoparticle aggregates comprising OPN and calcium phosphate for reducing or preventing bacterial biofilms formed by *Streptococcus spp*. and/or *Actinomyces spp*.

Another aspect of the present invention relates to the use of nanoparticle aggregates comprising OPN and strontium/calcium phosphate for reducing or preventing bacterial biofilms formed by *Streptococcus spp*. and/or *Actinomyces spp*.

Still another aspect relates to the use of nanoparticle aggregates comprising OPN and strontium phosphate for reducing or preventing bacterial biofilms formed by *Streptococcus spp.* and/or *Actinomyces spp*.

Yet another aspect relates to the use of nanoparticle aggregates comprising OPN and mixtures of strontium phosphate and calcium phosphate for reducing or preventing bacterial biofilms formed by *Streptococcus spp*. and/or *Actinomyces spp*.

Another aspect relates to the use of nanoparticle aggregates comprising a) OPN and b) strontium phosphate, calcium phosphate and mixtures of such, for reducing or preventing bacterial biofilms formed by *Streptococcus spp*. and/or *Actinomyces spp*.

Yet another aspect relates to the use of nanoparticle aggregates comprising OPN and calcium phosphate for reducing or preventing bacterial adhesion of *Streptococcus* spp. and/or *Actinomyces* spp.

Many problems occur in connection with care of the teeth, cosmetically as well as therapeutically, such as formation of dental biofilm (plaque), staining of teeth due to bacterial products, formation of dental calculus (tartar) dental caries, root canal infections and periodontal disease.

Dental plaque is a complex biofilm that accumulates on the hard tissues (teeth) in the oral cavity. Although dental biofilms harbour over 500 bacterial species, colonization follows a regimented pattern with adhesion of initial colonizers to the enamel salivary pellicle followed by secondary colonization through bacterial co-adhesion. It is well known that a range of *Streptococcus* species and *Actinomyces* species belong to the early colonizers. It is therefore important to control the adhesion and subsequent biofilm formation of these bacteria. A variety of adhesins and receptors are involved in bacterial adhesion to saliva-coated surfaces, in bacterial coaggregation, in bacterium-matrix interactions and contribute to biofilm development and ultimately to diseases such as caries, endodontic infections and periodontal disease.

One aspect relates to the use of nanoparticle aggregates comprising OPN and calcium phosphate for reducing or preventing oral biofilm growth.

When used to treat, prevent or reduce oral biofilm it is preferred that the nanoparticle aggregates are administered orally. It is furthermore preferred that the nanoparticle aggregates are present in a formulation suitable for oral administration.

Another aspect of the present invention relates to the use of nanoparticle aggregates comprising OPN and strontium/calcium phosphate for reducing or preventing oral biofilm growth.

Still another aspect relates to the use of nanoparticle aggregates comprising OPN and strontium phosphate for reducing or preventing oral biofilm growth.

Yet another aspect relates to the use of nanoparticle aggregates comprising OPN and mixtures of strontium phosphate and calcium phosphate for reducing or preventing oral biofilm growth.

Another aspect relates to the use of nanoparticle aggregates comprising a) OPN and b) strontium phosphate, calcium phosphate and mixtures of such, for reducing or preventing oral biofilm growth.

Still another aspect relates to the use of nanoparticle aggregates comprising a) OPN and b) strontium phosphate, calcium phosphate and mixtures of such, for reducing or preventing oral biofilm adhesion.

In one embodiment, the nanoparticle aggregates comprising OPN and calcium phosphate have the stoichiometric formula $A'_{10-X}A_X(PO_4)_{6-Y}B_Y(OH)_{2-z}C_z \cdot OPN_m \cdot (H_2O)_n$; wherein A' is selected from the group consisting of Ca, Sr and mixtures thereof; wherein A is selected from the group consisting of Li, Na, K, Rb, Cs, Be, Mg, Zn, Ba, H_2O, vacancy and mixtures thereof, and X=0-9; wherein B is selected from the group consisting of (CO_3), (SO_4), (HSO_4), (HPO_4), (H_2PO_4), H_2O, vacancy and mixtures thereof, and Y=0-5; wherein C is selected from the group consisting of F, Cl, Br, I, (CO3), H_2O, vacancy and mixtures thereof, and Z=0-2; wherein n=0-10 and m>0; wherein the molar ratio between (PO_4) and (HPO_4) is above 2.5.
Another aspect of the invention pertains to a method of curing, for alleviating and/or preventing a biofilm-related disease of an animal or human subject by administering to the subject nanoparticle aggregates as defined herein.

An aspect of the present invention relates to the use nanoparticle aggregates as defined herein for reducing, removing and/or preventing bad breath.

A further aspect of the invention pertains to a method of reducing or preventing microbial biofilm growth in or on an animal or human subject by administering to the subject nanoparticle aggregates as defined herein.

As stated above, the biofilm to be treated, reduced or prevented may e.g. be an oral biofilm, in which case oral administration of the nanoparticle aggregates is preferred.

The animal subject may e.g. be a domesticated animal such as a domesticated mammal, a domesticated fish or a domesticated bird.

A further aspect of the invention pertains to a method of reducing, removing and/or preventing bad breath of animal or human subject by administering to the subject nanoparticle aggregates as defined herein. The nanoparticle aggregates are preferably administered orally.

Yet an aspect of the invention pertains to the use of nanoparticle aggregates comprising a) OPN and b) a first particle comprising calcium and/or strontium, for reducing or preventing microbial biofilm growth. In some embodiments of the invention, it is required that this use is not a treatment of the human or animal body by therapy.

The biofilm can in theory be any of the biofilms mentioned herein, and may for example contain one more of the following bacteria: *Streptococcus spp., Staphylococcus spp., Pseudomonas spp. Actinomyces spp., Lactobacillus spp., Aggregatibacter spp., Bacteroides spp., Listeria spp., Campylobacter spp., Eikenella spp., Porphyromonas spp., Prevotella spp., Treponema spp.*
However, in some embodiments of the invention, the above use is not a treatment of the human or animal body by therapy. The biofilm may for example be a biofilm which is not in contact with a living human or a living animal.

Yet another aspect of the present invention pertains to a dental formulation comprising nanoparticle aggregates as defined herein.

The dental formulation may for example comprise the nanoparticle aggregates having the stoichiometric formula
$A'_{10-X}A_X(PO_4)_{6-Y}B_Y(OH)_{2-z}C_z \cdot OPN_m \cdot (H_2O)_n$; wherein A' is selected from the group consisting of Ca, Sr and mixtures thereof; wherein A is selected from the group consisting of Li, Na, K, Rb, Cs, Be, Mg, Zn, Ba, H_2O, vacancy and mixtures thereof, and X=0-9; wherein B is selected from the group consisting of (CO_3), (SO_4), (HSO_4), (HPO_4), (H_2PO_4), H_2O, vacancy and mixtures thereof, and Y=0-5; wherein C is selected from the group consisting of F, Cl, Br, I, (CO3), H_2O, vacancy and mixtures thereof, and Z=0-2; wherein n=0-10 and m>0; wherein the molar ratio between (PO_4) and (HPO_4) is above 2.5.

The dental formulations can be any dentifrice or related product of relevance in oral hygiene, such as for example toothpowder, tooth gel, tooth varnish, dental mouthwash, mouth spray or chewing gum.

In one embodiment, the dental formulation is in the form of a toothpaste, toothpowder, tooth gel, tooth varnish, dental mouthwash, mouth spray or chewing gum.

As disclosed in WO 2005/053,628, the amount of osteopontin is normally between about 50 mg OPN and about 1500 mg osteopontin per kg dental formulation, and that smaller amounts will also have an effect. Higher amounts can be used, but the effect will not be essentially increased. A useful amount is 100-1000 mg OPN per kg, preferably 200-500 mg, and most preferred about 350 mg. Higher amounts will presumably not give better results and are therefore not recommended, because OPN is a rather expensive ingredient. Surprisingly, nanoparticle aggregates comprising OPN and calcium phosphate have been shown by the inventors of the present invention to have a synergistic effect at reducing or preventing microbial biofilm growth. Therefore, the present invention reduces the effective amount of osteopontin per kg dental formulation.

In the present context, the term "oral biofilm growth" means biofilm growth on oral hard and soft tissues (oral mucosa, tongue, tooth surfaces) and biofilm growth on materials inserted in the oral cavity (implants, orthodontic brackets, and restorative materials such as fillings, crowns and dentures).

Preferred compositions of the subject invention are as already mentioned in the form of tooth-pastes, tooth varnish, tooth-gels and tooth powders. Components of such toothpaste and tooth-gels include one or more of the following: a dental abrasive (from about 10 % to about 50 %), a surfactant (from about 0.5 % to about 10 %), a thickening agent (from about 0.04 % to about 0.5 %), a humectant (from about 0.1 % to about 3 %), a flavouring agent (from about 0.04 % to about 2 %), a sweetening agent (from 0.1% to about 3 %), a colouring agent (from about 0.01 % to about 0.5 %) and water (from 2 % to 45 %).

Unless it is stated otherwise, the percentages of the components which form part of the compositions or products of the present invention are weight percent relative to the total weight of the composition or product.

Caries controlling agents may contain from 0.001 % to about 1 % nanoparticle aggregates comprising OPN and calcium phosphate. Anti-calculus agents contain from about 0.1 % to about 13 % nanoparticle aggregates comprising OPN and calcium phosphate.

Tooth powders, of course, are substantially free from all liquid components.

Other preferred compositions of the subject invention are dental mouth washes, including mouth sprays. Components of such mouth washes and mouth sprays typically include one or more of the following: water (from about 45 % to about 95 %), ethanol (from about 0 % to about 25 %), a humectant (from about 0 % to about 50 %), a surfactant (from about

0.01 % to about 7 %), a flavouring agent (from about 0.04 % to about 2 %), a sweetening agent from (from about 0.1 % to about 3 %), and a colouring agent (from about 0.001 % to about 0.5 % anti-caries agent including nanoparticle aggregates comprising OPN and calcium phosphate, from about 0.001 % to 1 % and an anti-calculus agent (from about 0.1 % to about 13 %).

A third area of application is in chewing gum formulations of various compositions in general terms.

Strontium (Sr) has been reported to promote bone formation and is approved for the treatment of osteoporosis.

Still another aspect relates to a coating composition comprising nanoparticle aggregates as defined herein.

The coating composition may for example contain nanoparticle aggregates having the stoichiometric formula $A'_{10-X}A_X(PO_4)_{6-Y}B_Y(OH)_{2-z}C_z \cdot OPN_m \cdot (H_2O)_n$; wherein A' is selected from the group consisting of Ca, Sr and mixtures thereof; wherein A is selected from the group consisting of Li, Na, K, Rb, Cs, Be, Mg, Zn, Ba, H_2O, vacancy and mixtures thereof, and X=0-9; wherein B is selected from the group consisting of (CO_3), (SO_4), (HSO_4), (HPO_4), (H_2PO_4), H_2O, vacancy and mixtures thereof, and Y=0-5; wherein C is selected from the group consisting of F, Cl, Br, I, (CO3), H_2O, vacancy and mixtures thereof, and Z=0-2; wherein n=0-10 and m>0; wherein the molar ratio between (PO_4) and (HPO_4) is above 2.5.

The coating composition may e.g. comprise nanoparticle aggregates comprising OPN and calcium phosphate.

The coating composition may be used in connection with bone disease, bone fracture or implants.

One aspect relates to the use of the coating compositions according to the present invention to coat medical devices.

Another aspect relates to a food or bevarage product comprising nanoparticle aggregates as defined herein.

The food or bevarage product may for example comprise nanoparticle aggregates having the stoichiometric formula

$A'_{10-X}A_X(PO_4)_{6-Y}B_Y(OH)_{2-Z}C_Z \cdot OPN_m \cdot (H_2O)_n$; wherein A' is selected from the group consisting of Ca, Sr and mixtures thereof; wherein A is selected from the group consisting of Li, Na, K, Rb, Cs, Be, Mg, Zn, Ba, H_2O, vacancy and mixtures thereof, and X=0-9; wherein B is selected from the group consisting of (CO_3), (SO_4), (HSO_4), (HPO_4), (H_2PO_4), H_2O, vacancy and mixtures thereof, and Y=0-5; wherein C is selected from the group consisting of F, Cl, Br, I, (CO3), H_2O, vacancy and mixtures thereof, and Z=0-2; wherein n=0-10 and m>0; wherein the molar ratio between (PO_4) and (HPO_4) is above 2.5.

An object of the present invention is to improve the reduction of biofilm on surfaces.

One aspect relates to a product comprising a bulk part and a surface region; wherein a first surface region coating is coated on at least a first part of said surface region; said first surface region coating comprising nanoparticle aggregates as defined herein.

The first surface region coating may e.g. comprise nanoparticle aggregates having the stoichiometric formula
$A'_{10-X}A_X(PO_4)_{6-Y}B_Y(OH)_{2-Z}C_Z \cdot OPN_m \cdot (H_2O)_n$; wherein A' is selected from the group consisting of Ca, Sr and mixtures thereof; wherein A is selected from the group consisting of Li, Na, K, Rb, Cs, Be, Mg, Zn, Ba, H_2O, vacancy and mixtures thereof, and X=0-9; wherein B is selected from the group consisting of (CO_3), (SO_4), (HSO_4), (HPO_4), (H_2PO_4), H_2O, vacancy and mixtures thereof, and Y=0-5; wherein C is selected from the group consisting of F, Cl, Br, I, (CO3), H_2O, vacancy and mixtures thereof, and Z=0-2; wherein n=0-10 and m>0; wherein the molar ratio between (PO_4) and (HPO_4) is above 2.5.

In one embodiment, the surface region comprises a material selected from the group consisting of metals, metal oxides, inorganic materials, organic materials, and polymers.

In another embodiment, the surface region is positively charged.

In yet another embodiment, the surface region is negatively charged.

In still another embodiment, the surface region is uncharged.

In the present context, the term 'anti-biofilm agent' is to be understood as an agent that prevents and/or reduces microbial adhesion to surfaces and/or prevents and/or reduces microbial biofilm formation and/or disrupts and/or destabilizes microbial biofilms.

One aspect of the invention relates to an anti-biofilm agent comprising a) OPN and b) a first particle comprising calcium and/or strontium.

The anti-biofilm agent may for example comprise nanoparticle aggregates comprising a) OPN and b) strontium phosphate, calcium phosphate or mixtures of such.

The anti-biofilm agent may e.g. comprise nanoparticle aggregates having the stoichiometric formula $A'_{10-X}A_X(PO_4)_{6-Y}B_Y(OH)_{2-Z}C_Z \cdot OPN_m \cdot (H_2O)_n$; wherein A' is selected from the group consisting of Ca, Sr and mixtures thereof; wherein A is selected from the group consisting of Li, Na, K, Rb, Cs, Be, Mg, Zn, Ba, H_2O, vacancy and mixtures thereof, and X=0-9; wherein B is selected from the group consisting of (CO_3), (SO_4), (HSO_4), (HPO_4), (H_2PO_4), H_2O, vacancy and mixtures thereof, and Y=0-5; wherein C is selected from the group consisting of F, Cl, Br, I, (CO3), H_2O, vacancy and mixtures thereof, and Z=0-2; wherein n=0-10 and m>0; wherein the molar ratio between (PO_4) and (HPO_4) is above 2.5.

Another aspect of the invention relates to a method for producing nanoparticle aggregates formed by OPN and calcium phosphate comprising:

a) Providing a first aqueous solution comprising phosphate as PO_4^{3-}; wherein the pH is within the range of 6-14;

b) Providing a second aqueous solution comprising Ca^{2+} and/or Sr^{2+}; wherein the pH is within the range of 6-14;

wherein either the first, second or both aqueous solutions comprises OPN;

c) Mixing said first and second solutions, thereby forming a suspension comprising nanoparticle aggregates comprising 1) OPN, 2) strontium phosphate, calcium phosphate or mixtures of such, and 3) water soluble electrolytes;

d) Optionally, removing a substantial amount of said water soluble electrolytes from the suspension;

e) Optionally, separating said nanoparticle aggregates from the water phase.

In the present context, the term 'aqueous solution' is to be understood as a liquid matter comprising at least 50% w/w of water.

In the present context, the term 'suspension' is to be understood as a heterogeneous fluid containing solid particles that are sufficiently large for sedimentation.

In the present context, the term 'electrolyte' is to be understood as a substance containing free ions that make the substance electrically conductive. In the present invention, the electrolyte must be soluble in water.

In one embodiment, the pH of the water phase of the suspension is above 7, such as within the range of 7.4-14.0, e.g. above 7.6, such as within the range of 7.8-13.5, e.g. above 8.0, such as within the range of 8.5-13.0, e.g. above 9.0, such as within the range of 9.5-12.5, e.g. above 10.0, such as within the range of 10.5-12.0, e.g. above 11.0.

In one embodiment, the process further comprises the step d).

In another embodiment, the process further comprises the step d), wherein the separation is performed by dialysis.

In the present context, the term 'dialysis', is to be understood as separation of suspended colloidal particles from dissolved ions or molecules of small dimensions (crystalloids) by means of their unequal rates of diffusion through the pores of semipermeable membranes.

In one embodiment, the process further comprises the step e).

In another embodiment, the total concentration of osteopontin in the first and second aqueous solutions is above 2.5 mg/ml, such as within the range of 3-1000 mg/ml, e.g. above 5 mg/ml, such as within the range of 10-500 mg/ml, e.g. above 12 mg/ml, such as within the range of 15-100 mg/ml, e.g. above 18 mg/ml, such as within the range of 20-50 mg/ml, e.g. above 25 mg/ml, such as within the range of 30-45 mg/ml, e.g. above 35 mg/ml.

In one embodiment, the process further comprises the step f) sterilizing the obtained nanoparticle aggregates.

In another embodiment, the process further comprises the step f), wherein the sterilization step is performed by heating.

In yet another embodiment, the process further comprises the step f), wherein the sterilization step is performed by heating to 80 °C and keeping the temperature at this level for 1 hour or more.

The inventors have found that there is an upper limit of the osteopontin concentration of about 33 wt% of dried mass (i.e. referring to the mass obtained by drying at 200 °C), where no more osteopontin seems to be included in the nanoparticle aggregates. This sets in at an added OPN amount of about 20 mg/l. Hence, in one embodiment, the total concentration of osteopontin in the first and second aqueous solutions is within the range of 2.5-35 mg/ml. However, this limit may change depending on the concentration of different water soluble electrolytes.

In one embodiment, the osteopontin is only present in the first aqueous solution.

In another embodiment, the osteopontin is only present in the second aqueous solution.

In yet another embodiment, the osteopontin is present in both the first and the second aqueous solution.

In one embodiment, step c) is performed at a temperature within the range of 5-80 degrees Celsius, such as within the range of 10-50 °C, e.g. within the range of 20-40 °C, such as within the range of 22-28 °C.

In one embodiment, either the first, second or both aqueous solutions comprises a Ca/Sr-binding fluorescent dye. An additional Ca/Sr-binding dye in the solutions will result in fluorescent (if fluorescent dye) or colored nanoparticle aggregates.

It should be noted that embodiments and features described in the context of one of the aspects of the present invention also apply to the other aspects of the invention.

All patent and non-patent references cited in the present application, are hereby incorporated by reference in their entirety.

The invention will now be described in further details in the following non-limiting examples.

Examples

Example 1: Synthesis of nanoparticle aggregates

For synthesis of nanoparticle aggregates, the following method was used

1) Three aqueous solutions were prepared in equal volumes, one containing 0.36 M Na_3PO_4 (conveniently made by making a solution that was 0.36 M NaH_2PO_4 and 0.72 M NaOH), the second containing 0.6 M $CaCl_2$ and the third containing three times the desired final amount of OPN.
2) The $CaCl_2$ and OPN solutions were mixed first, and then the Na_3PO_4 solution was added. This mixing produced a turbid gel-like suspension. The gel-like structure broke down under stirring on a magnet stirrer. The solution was stirred for 24 h at 25 °C using a custom designed temperature controlled water bath.
3) After 24 h, the solution was transferred to dialysis bags to remove excess NaCl. The dialysis was carried out for 24 h in a reservoir containing 100 times the solution volume of water that was slowly stirred by a magnetic stirrer. The reservoir water was changed after 12 h of dialysis.
4) After dialysis the nanoparticle aggregates were centrifuged at 4800 rpm and washed with water two times.
5) After washing, the nanoparticle aggregates were dried at 60 °C before analysis.

The results discussed below were obtained by reaction (step 2 above) at 25 °C; the reaction can equally well be performed at other temperatures both higher and lower. At increased temperatures the reaction is faster. The synthesis has also been executed using K_3PO_4 as the phosphate source. The reaction can also be completed with lower (higher) concentrations of reagents with ensuing smaller (higher) yields.

Samples with different amounts of OPN were produced. Samples containing 0, 0.001, 0.0025, 0.01, 0.025, 0.1, 0.25, 1.0, 2.5, 5.0, 10.0, 12.5, 15.0, 20.0, 25.0, 30.0 and 34.0 mg/mL were synthesised (17 different concentrations in total).

Nanoparticle aggregates for biofilm experiments were synthesized with an OPN amount added of 12.5 mg/ml as described above with the modification that the dialysis reservoir liquid had pH and 0.9 wt% NaCl. After 24 h dialysis, the resulting suspension was sterilized by heating the suspension in a closed container at 80 °C for 1 h. The above steps 4) and 5) were omitted in the production of the nanoparticle aggregates for biofilm experiments.

The nanoparticle aggregates were characterized by XRD, FTIR and TGA. For XRD and FTIR measurements, the dry particles were ground to fine powder before measurement. XRD was measured on a Rigaku SmartLab with a Bragg-Brentano setup. Parameters for the measurements were: 6-120° 2θ, step size 0.02, 4°/min. Two scans were performed and averaged for each sample. Figure 9 shows a selected segment of the combined data. Rietveld refinements were performed for all samples with sufficient crystallinity. Samples with 20 mg/mL or higher OPN content were not refined, as these samples had a high amorphous content which complicates the Rietveld refinements. For the samples which were Rietveld refined, selected parameters were extracted and are presented in Figure 10. FTIR was conducted on a Nicolet 380 FT-IR, Smart Orbit (Thermo electron corporation). Samples were dried at 60 °C just before measuring. A background was fitted for each individual spectrum and subtracted. The corrected data can be seen in Figure 13. Integration of specific peaks were made for comparison between phosphate (1200-900 cm^{-1}), carbonate (890-840 cm^{-1}) and organic content (1720-1595 cm^{-1}) of the samples. Comparisons between FTIR mineral:organic and mineral:carbonate peak ratios are shown in Figure 14.

TGA data were recorded on a Netzsch STA 449 C (NETZSCHGeratebau GmbH, Selb, Germany) using an atmosphere of Ar and O_2. TGA data are shown in Figure 11 while extracted mass losses are shown in Figure 12.

The XRD data showed that crystalline material formed for all samples with lower than 20 mg/ml OPN. At and above 20 mg/ml a very large amount of amorphous material was observed. At 30 and 34 mg/ml minor peaks were observed atop of the amorphous scattering. The diffraction peaks for the crystalline materials were significantly broader than the experimental resolution reflecting that the crystalline materials are nanocrystalline. The X-ray diffraction data up to and including 15 mg/ml OPN were modelled by Rietveld refinement to extract information on average nanocrystals sizes. The nanocrystals are anisotropic in size and needle shaped with the long morphological axis coinciding with the crystallographic c-axis. Extracted crystallite sizes are shown in Figure 10. At very low OPN contents, there was a rapid drop in crystallite size in both morphological directions (bottom plot in Figure 10) after which the sizes remained almost constant up to an OPN content of ~1 mg/ml after which it again decreased up to an OPN content of 10 mg/ml. For 12.5 and 15 mg/ml the particle aggregates contained larger nanocrystals than for 10 mg/ml but comparison between background and peak heights showed that there was a large amount of amorphous material in these samples. The content of water, organic components and carbonate was assessed by TGA measurements as shown in Figure 11. Mass losses are reported in Figure 12; the mass losses

corresponding to organic and carbonate contents were corrected to dry mass (i.e. normalized by mass remaining after the initial loss of water). The water content was in all cases below 15 wt%. It was significantly larger for the amorphous compounds (~10 wt%) than for the noncrystalline materials. For the nanocrystalline materials, a linearly increasing water content with OPN content was observed following 3.1+0.12·OPN, where OPN is the OPN added in mg/ml. The carbonate content was ~2.4 dry wt% for the nanocrystalline and 4.8 dry wt% for the amorphous materials, respectively. The organic content increased with OPN added up to 10 mg/ml with 12.5 and 15 mg/ml displaying smaller organic content; at high concentrations the organic content saturated at ~33 dry wt%. FTIR confirmed the observations above as show in Figure 13 and 14.

Example 2: Growth of dental caries model biofilms

The human oral bacterial isolates *Streptococcus oralis* SK248, *Streptococcus downei* HG594, *Streptococcus mitis* SK24, *Streptococcus sanguinis* SK150 and *Actinomyces naeslundii* AK6 were used in the experiments. Organisms were cultivated aerobically on blood agar (SSI, Copenhagen, Denmark) and transferred to THB (Roth, Karlsruhe, Germany) at 35 °C until mid to late exponential phase prior to experimental use.

Flow cells (ibiTreat, µ-slide VI, Ibidi, Munich, Germany) were preconditioned with 1/10 THB (pH 7.0). Bacterial cultures, adjusted to an optical density of 0.4 at 550 nm (corresponding to 2-5*10^9 cells/ml), were inoculated sequentially into the flow channels in the following order: 1. *S. oralis* SK248; 2. *A. naeslundii* AK6; 3. *S. mitis* SK24; 4. *S. downei* HG594; 5. *S. sanguinis* SK150. 0.4 ml of each organism was injected through the silicone tubing at the in-port using sterile needles (BD Microlance, 27G, Drogheda, Ireland), and injection holes were sealed with silicone glue (Dow Corning, Wiesbaden, Germany). Following injection, the flow was halted for 30 min to allow for bacterial adhesion. Nonadherent organisms were removed by 10 min of flow prior to injection of the next organism. After inoculation procedure, biofilms were grown for 26 h at 35 °C under constant flow (250 µl/min; 28.3 mm/min) of 1/10 THB (pH 7.0) provided by a peristaltic pump (Watson Marlow 205 U, Wilmington, Massachusetts, USA).

Example 3: Binding of OPN to bacteria in caries model biofilms

OPN was labelled with fluorescein according to the manufacturer's instructions (Invitrogen, Taastrup, Denmark). After growth phase, biofilms were incubated for 45 min with 100 µl of the labelled protein at 35 °C and imaged using the 488 nm laser line and a 500-550 nm band pass filter. XY resolution was set to 0.1 µm/pixel and Z resolution corresponded to 1 Airy unit (0.8 µm optical slice thickness) (Figure 1A).

Example 4: Binding of calcium phosphate nanoparticle aggregates containing OPN to *in vivo* grown dental biofilm

In vivo grown dental biofilm was scraped from a tooth surface with a sterile curette and collected on a glass slab (4x4x1 mm). The glass slab was turned upside down, and the biofilm was incubated with calcium phosphate nanoparticle aggregates containing OPN for 30 min at 35 °C. After washing with 0.9% NaCl, the biofilm was stained with 30 µM C-SNARF-4 (Sigma-Aldrich, Brøndby, Denmark) that targets both bacteria in the biofilms and nanoparticle aggregates. A Zeiss LSM 510 META (Jena, Germany) with a 63x oil immersion objective, 1.4 numerical aperture (Plan-Apochromat) was used for image acquisition. The probe was exited with a 543 nm laser line (250-300 µW), and fluorescence emission was monitored within 576-608 nm interval (META detector), with the pin hole set to 2 Airy units (1.6 µm optical slice thickness). Images were 364x364 pixels (143x143 μm^2) in size and were acquired with pixel dwell time 18 µs, line average 2, 0.4 µm/pixel (zoom 1), 12-bit intensity resolution (Figure 1B).

Example 5: Effect of OPN on bacterial growth in planktonic culture

Bacteria were transferred to THB or THB containing 26.5 µmol/l OPN. Aliquots of 100 µl were transferred to a 96 well plate (Sarstedt, Newton, NC, USA) and OD at 550 nm was measured with a spectrophotometer (BioTek PowerWave XS2, Bad Friedrichshall, Germany). Experiments were carried out in triplicates and repeated once (Figure 2).

Example 6: Effect of calcium phosphate nanoparticle aggregates containing OPN on biofilm growth in the caries model

Biofilms were grown as described above and treated three times with calcium phosphate nanoparticle aggregates containing OPN during growth (3h, 9h and 24h after inoculation procedure). Nanoparticle aggregate suspensions obtained from Example 1 were shaken vigorously, set to settle for 10 min, and 0.4 ml were aspirated from the top of the suspensions. The aspirated suspension had a content of nanoparticle aggregates of approx. 3% (w/v). Then the flow was halted and the aspirated suspension including nanoparticle aggregates was injected into the channels as described for the bacterial inocula. After one hour of incubation the flow was started again. Control treatments were performed in the same way with osteopontin-free calcium phosphate nanoparticle aggregates, silicium dioxide particles of different sizes (100 nm, 500 nm and 2000 nm diameter; 3.5% (w/v) in 0.9% NaCl; Sigma-Aldrich, Brøndby, Denmark), polystyrene particles (1000 nm diameter; 3.5% (w/v) in distilled water; Sigma-Aldrich), and dissolved OPN (0.9 g/l in 0.9% NaCl). Channels incubated with 0.9% NaCl served as negative controls. Six replicate biofilms

were grown for each experimental setting. After biofilm growth, THB was removed from the flow channels by aspiration with paper points. The channels were rinsed with distilled water, dried again and stained with 100 µL of 2% crystal violet solution for 1 h. Then channels were rinsed again with distilled water, dried, and 120 µL of 100% ethanol (Sigma-Aldrich, Brøndby, Denmark) were added during 30 min to destain the biofilms. Thereafter, 100 µL of the stained ethanol solutions, diluted 1:8, were transferred to a 96 well plate (Sarstedt, Newton, NC, USA), and optical density at 585 nm was measured with a spectrophotometer (BioTek PowerWave XS2, Bad Friedrichshall, Germany). Empty flow channels were processed in the same way and used for background subtraction (Figure 3).

Figure 3 shows the effect of different agents on biofilm formation in the caries model, measured by crystal violet staining. Calcium phosphate nanoparticle aggregates containing OPN (HAP-OPN) strongly reduce the amount of biofilm formed in the flow cells, as compared to 1000 nm polystyrene particles, silica particles (150 nm, 500 nm and 2000 nm), OPN-free calcium phosphate particle aggregates and 0.9 g/l OPN.

Thus, Example 6 provides statistically significant evidence demonstrating that one obtains a synergetic anti-biofilm effect by using OPN bound to calcium-containing particles.

Example 7: Crystal violet binding of calcium phosphate nanoparticle aggregates containing OPN

100 µl of calcium phosphate nanoparticle aggregates containing OPN were injected into flow cells (ibiTreat, µ-slide VI, Ibidi, Munich, Germany) and set to dry overnight. Flow channels were stained with crystal violet for 15 min, washed twice with PBS, dried and filled with 120 µl of 100% ethanol for 15 min. 100 µL of the stained ethanol solutions, diluted 1:64, were transferred to a 96 well plate and optical density at 585 nm was measured. Osteopontin-free calcium phosphate nanoparticle aggregates, silicium dioxide particles (500 nm diameter; 3.5% (w/v) in 0.9% NaCl), polystyrene particles (1000 nm diameter; 3.5% (w/v) in distilled water), dissolved OPN (0.9 g/l in 0.9% NaCl) and 0.9 g/l NaCl served as controls. Experiments were performed in triplicates (Figure 4).

Example 8: Effect of calcium phosphate nanoparticle aggregates containing OPN on oral biofilm formation *in vivo*

Oral biofilms were grown on custom-made glass slabs (4x4x1 mm) (Menzel, Braunschweig, Germany) with a surface roughness of 1200 grit. Glass slabs were mounted recessed in the buccal flanges of individually designed intraoral appliances. Two volunteers kept an appliance intraorally for 72 h, except during tooth brushing, intake of food or

liquids other than water and during nanoparticle aggregate dips. One side of the appliance was dipped 5-6 times (30-60 min) each day in a suspension containing 0.9% NaCl and approx. 3% (w/v) nanoparticle aggregates prepared in Example 1. At the same time, the other side of the appliance was dipped in 0.9% NaCl and served as negative control. After 72 h, glass slabs were removed and biofilms were stained with C-SNARF-4 prior to confocal microscopic analysis (Figure 6).

Figure 6 shows that calcium-containing nanoparticle aggregates containing OPN strongly reduce oral biofilm growth *in vivo*. A: Biofilm grown on a glass slab kept intraorally for 72 h. per day, 5-6 NaCl dips (30-60 minutes) were performed. B: Biofilm grown on a glass slab kept intraorally for 72 h.

Thus, Example 8 confirms that the anti-biofilm effect observed in Example 6 also exists *in vivo.*

Example 9: Effect of calcium phosphate nanoparticle aggregates containing OPN on pH of planktonic bacterial cultures

Bacterial cultures were grown in THB until mid-exponential phase, washed twice and transferred to sterile saliva. OD was adjusted to 1.0 (550 nm), 0.4% glucose (w/w) was added and 1 ml of bacterial suspension was mixed with 1 ml of calcium phosphate nanoparticle aggregates containing OPN or 1 ml of 0.9% NaCl. pH was measured for 20 h. Experiments were performed in duplicate and repeated once (Figures 7a-7e).

Example 10: Effect of calcium phosphate nanoparticle aggregates containing OPN on pH in caries model biofilms

Inoculation procedure was carried out as described above. Biofilms were then grown on 1/10 diluted THB for 8 h at 35 °C with a flow rate of 250 µL/min. By that time, stable thin biofilms had formed. Then the medium was changed to carbohydrate free beef extract (Scharlau, Barcelona, Spain) and the flow rate was reduced to 50 µL/min to minimize shear forces in the flow cell. 1 h, 12 h and 17 h after medium change, HAP-OPN particles were injected and incubated with the biofilms for 1 h as described above. Control channels were incubated with 0.9% NaCl.

Confocal microscopic calibration of the ratiometric pH-sensitive probe C-SNARF-4: HEPES buffer solutions (50 mM; adjusted to pH 4.5-8.5 in steps of 0.1 pH units), containing C-SNARF-4 at a concentration of 30 µM, were imaged in flow channels. A Zeiss LSM 510 META (Jena, Germany) with a 63x oil immersion objective, 1.4 numerical aperture (Plan-

Apochromat) was used for image acquisition. The probe was exited with a 543 nm laser line (250-300 µW), and fluorescence emission was monitored simultaneously within 576-608 nm (green) and 629-661 nm (red) intervals (META detector), with the pin hole set to 2 Airy units (1.6 µm optical slice thickness). Images were 364x364 pixels (143x143 μm^2) in size and were acquired with pixel dwell time 18 µs, line average 2, 0.4 µm/pixel (zoom 1), 12-bit intensity resolution. For every third pH value, a measurement was performed on unstained HEPES buffer (50 mM, pH 8.5) for background subtraction. Additionally, unstained solutions of glucose (20% w/v) and lactate (20% w/v), 1/10 diluted THB (pH 7), sterile saliva, PBS (pH 7.4, Sigma-Aldrich, Brøndby, Denmark), untreated biofilms and HAP-OPN particle suspensions were imaged with identical microscope and laser settings. No autofluorescent signals were emitted by any of these controls in the wavelength ranges 576-608 nm and 629-661 nm.

For ratio calculation, regions of 100 x 100 pixels were defined within each image and the average and standard deviations were determined using the LSM acquisition software. Subsequently, the ratio R, standard deviation s_R and standard error of mean, S_R, were calculated for each pH value according to equations (1), (2) and (3):

(1) $$R = \frac{g - b_g}{r - b_r}$$

(2) $$s_R = \frac{1}{\left((r-b_r)^2\right)^{1/2}} \left(s_g^2 + s_{bg}^2 + \frac{(g-b_g)^2}{(r-b_r)^2}\left(s_r^2 + s_{br}^2\right)\right)^{1/2}$$

(3) $$S_R = \frac{1}{\left(100^2\right)^{1/2}} \left(s_R^2\right)^{1/2}$$

g, r, s_g and s_r are the averages and standard deviations within the 100 x 100 pixels region defined in the respective green and red images. b_g, b_r, s_{bg} and s_{br} are the corresponding values for the background images. The resulting values of R were plotted in MATLAB (MathWorks, Natick, Massachusetts, US), and fitted to the function:

(4) $$pH = \ln\left(\frac{-1.6546}{R - 1.7469} - 1\right)\frac{1}{(2.3981)} + 5.9799$$

Measurements were performed twice and proved to be highly reproducible.

Biofilm pH imaging: Three biofilms were grown in parallel, one of which was treated with calcium phosphate nanoparticle aggregates containing OPN as described above. Thereafter, biofilms were washed twice with sterile saliva, and C-SNARF-4 was added to a concentration of 30 µM. The flow cell was transferred to the microscope, which was kept at 37 °C with an XL incubator (PeCon, Erbach, Germany), and baseline pH images were

acquired in the bottommost layer of the biofilms. Subsequently, glucose-free saliva was replaced by salivary solution containing 0.4 % (w/v) glucose and 30 µM of C-SNARF-4 in two of the three channels. In each of the three biofilms, pH images were acquired in 16 microscopic fields of view chosen at random. XY positions were marked in the LSM software, and 60 min after the addition of glucose, identical microscopic fields were imaged again in the same order. For background subtraction, images were acquired with the 543 nm laser switched off. The microscope and laser settings were identical to those of the calibration measurements. Five independent replicates of the experiments were performed (Figures 8a-8e).

Claims

1. Nanoparticle aggregates comprising a) osteopontin (OPN) and b) a first particle comprising calcium and/or strontium, for use as a medicament.

2. Nanoparticle aggregates comprising a) OPN and b) a first particle comprising calcium and/or strontium for curing, alleviating and/or preventing a biofilm-related disease.

3. Nanoparticle aggregates according to claim 2, wherein the biofilm-related disease involves a bacterial infection.

4. Nanoparticle aggregates according to claim 2 or 3, wherein the biofilm-related disease is an oral disease.

5. Nanoparticle aggregates according to claim 4, wherein the biofilm-related disease is dental caries.

6. Nanoparticle aggregates according to claim 4 or 5, wherein the biofilm-related disease is gingivitis.

7. Nanoparticle aggregates according to any of the claims 4-6, wherein the biofilm-related disease is periodontitis.

8. Nanoparticle aggregates according to claim 2 or 3, wherein the biofilm-related disease is a disease selected from the group consisting of bacterial endocarditis, chronic wound infections, implant infections, otitis media, cystic fibrosis, and a combination thereof.

9. Nanoparticle aggregates according to claim 2 for curing, alleviating and/or preventing a bacterial infection, e.g. a bacterial wound infection.

10. Nanoparticle aggregates comprising a) OPN and b) a first particle comprising calcium and/or strontium for reducing or preventing microbial biofilm growth or for removing microbial biofilm.

11. Nanoparticle aggregates according to claim 10, wherein the biofilm is dental plaque.

12. Nanoparticle aggregates according to any of the claims 2-11, wherein the biofilm contains bacteria having a OPN binding capacity of at least 50 OPN molecules per cell.

13. Nanoparticle aggregates according to any of the claims 2-12, wherein the biofilm contains, or even consists of, one or more bacteria selected from the group consisting of *Streptococcus spp., Staphylococcus spp., Pseudomonas spp. Actinomyces spp., Lactobacillus spp., Aggregatibacter spp., Bacteroides spp., Listeria spp., Campylobacter spp., Eikenella spp., Porphyromonas spp., Prevotella spp., Treponema spp., and combinations thereof.*

14. Nanoparticle aggregates according to any of the preceding claims, wherein the first particle comprises calcium.

15. Nanoparticle aggregates according to any of the preceding claims, wherein the first particle comprises strontium.

16. Nanoparticle aggregates according to any of the preceding claims, wherein the first particle comprises calcium and strontium.

17. Nanoparticle aggregates according to any of the preceding claims, wherein the first particle comprises an inorganic salt of calcium and/or strontium.

18. Nanoparticle aggregates according to claim 17, wherein the inorganic salt comprises a phosphate species, sulfate, and/or carbonate.

19. Nanoparticle aggregates according to any of the preceding claims, wherein the first particle comprises at least 50% (w/w) calcium phosphate.

20. Nanoparticle aggregates according to any of the preceding claims, wherein the first particle is capable of releasing calcium and/or strontium.

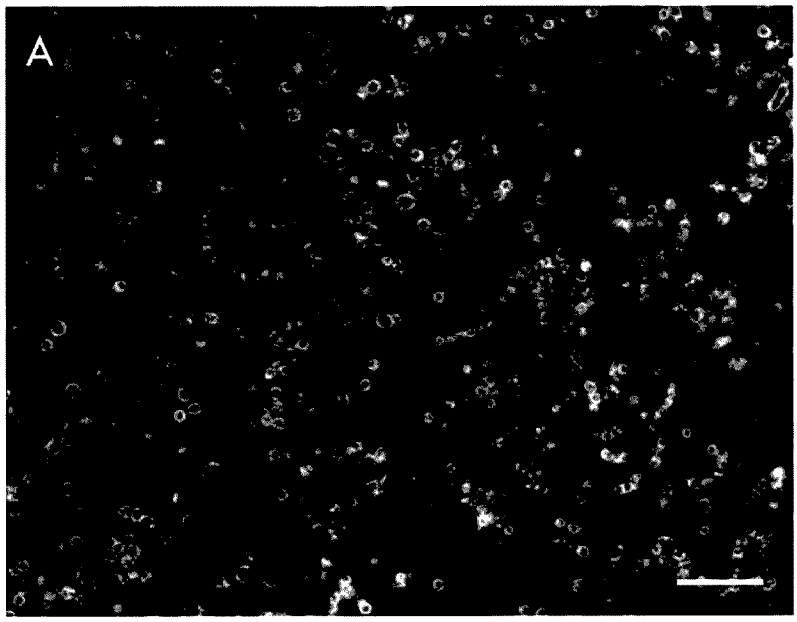
21. Nanoparticle aggregates according to any of the preceding claims, wherein the first particle is a nanoparticle.

22. Nanoparticle aggregates according to any of the preceding claims, comprising a second particle of the same type as the first particle.

23. Nanoparticle aggregates according to any of the preceding claims, having a hydrodynamic radius of at most 5 micron.

24. Use of nanoparticle aggregates comprising a) OPN and b) a first particle comprising calcium and/or strontium, for reducing or preventing microbial biofilm growth or for removing microbial biofilm.

25. Use according to claim 24, wherein the biofilm contains, or even consists of, one or more bacteria selected from the group consisting of *Streptococcus spp., Staphylococcus spp., Pseudomonas spp. Actinomyces spp., Lactobacillus spp., Aggregatibacter spp., Bacteroides spp., Listeria spp., Campylobacter spp., Eikenella spp., Porphyromonas spp., Prevotella spp., Treponema spp., and combinations thereof*.



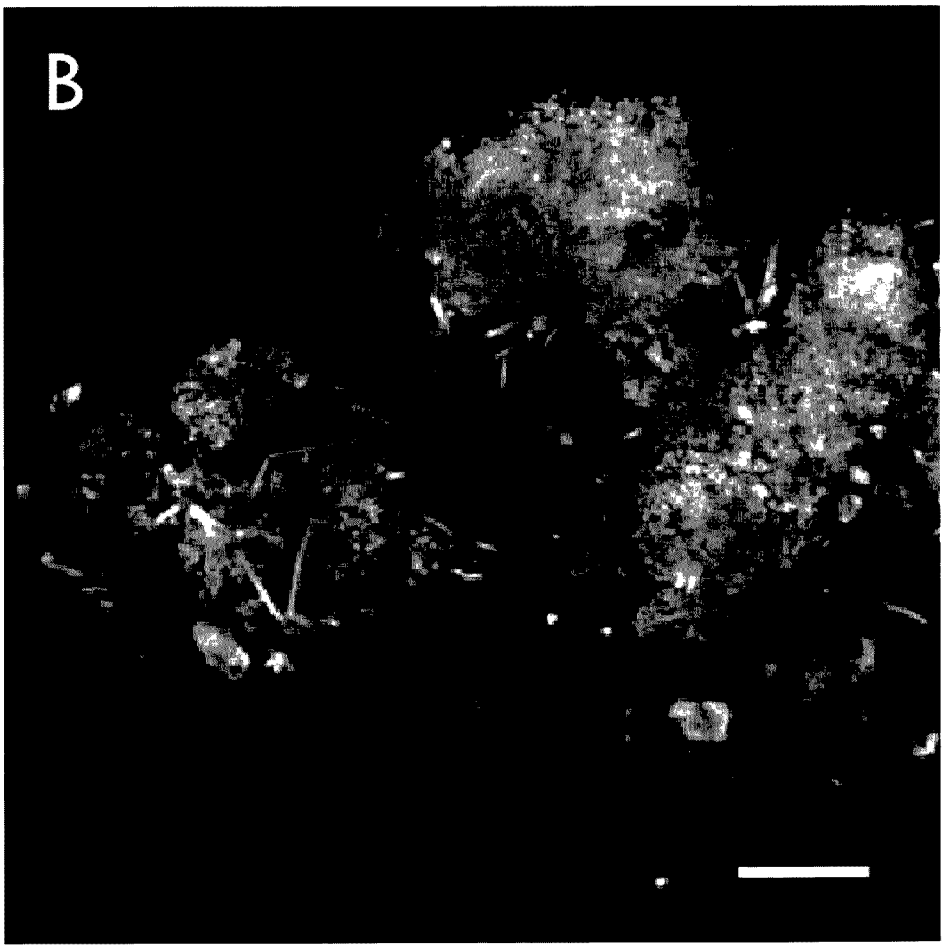
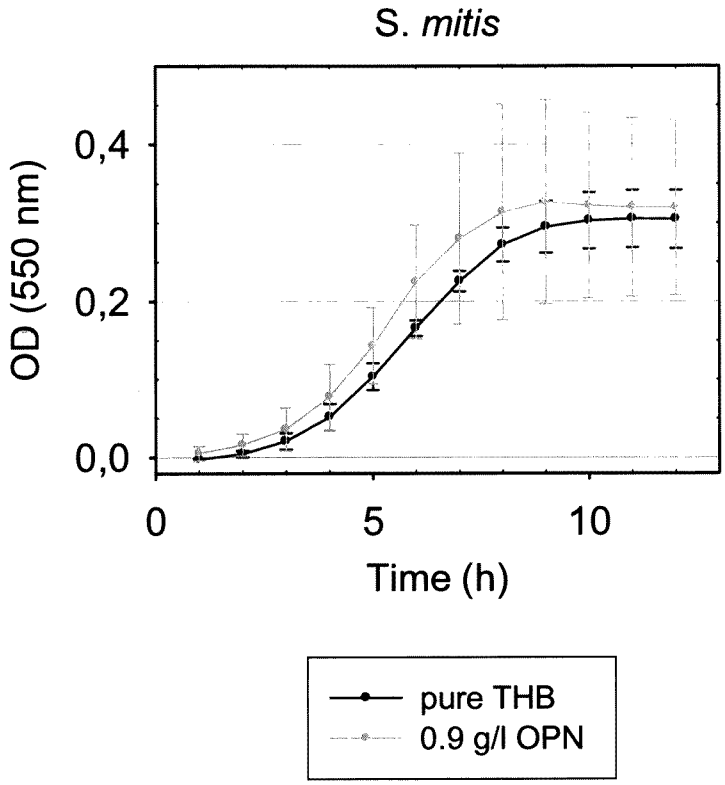
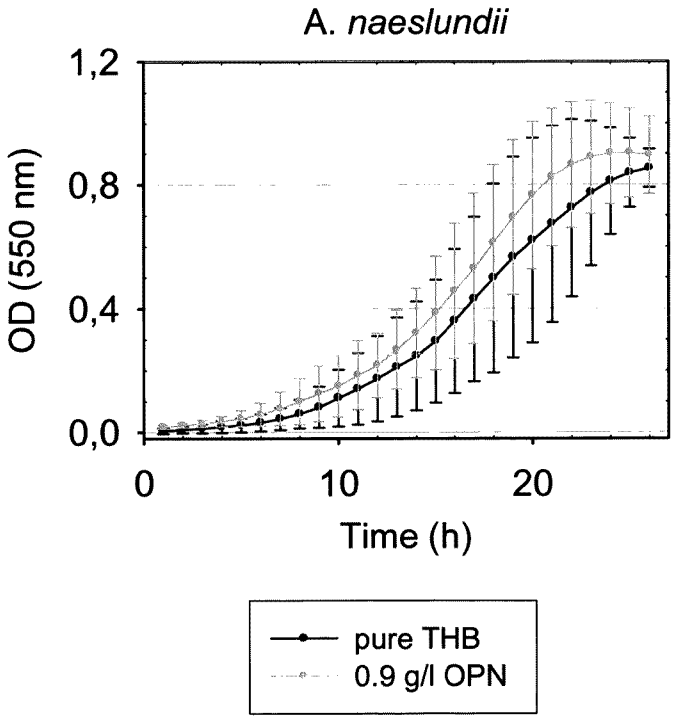


Figure 1



2A



2B

Figure 2

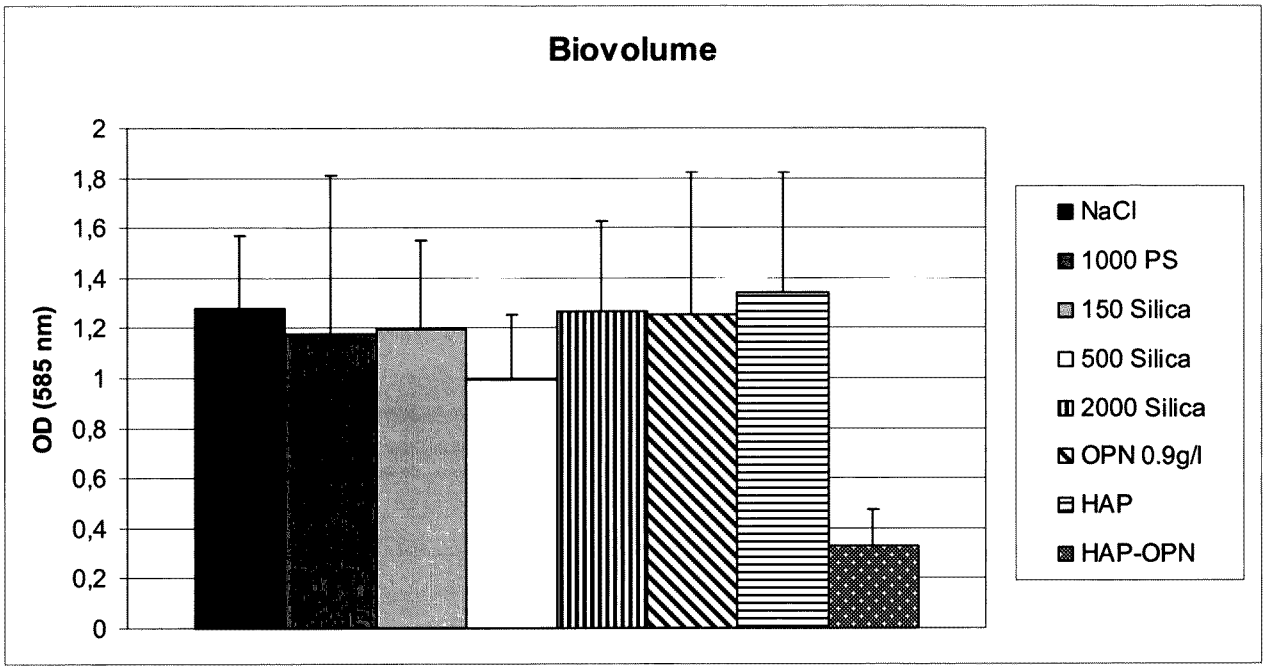


Figure 3

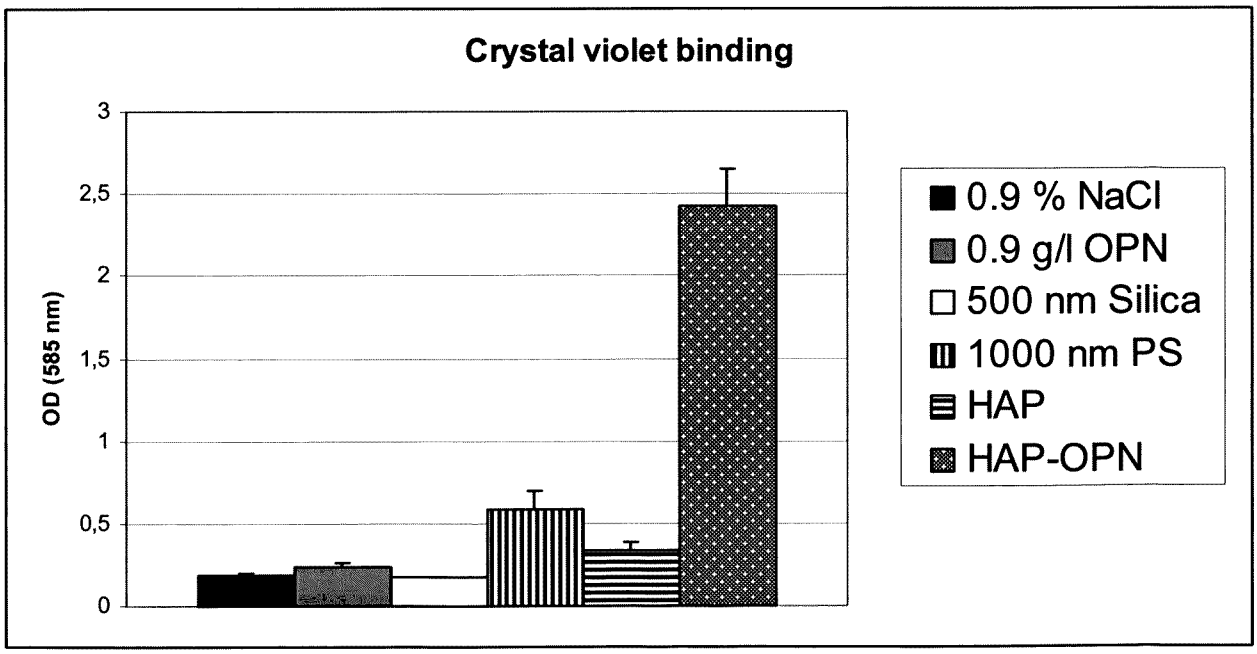


Figure 4

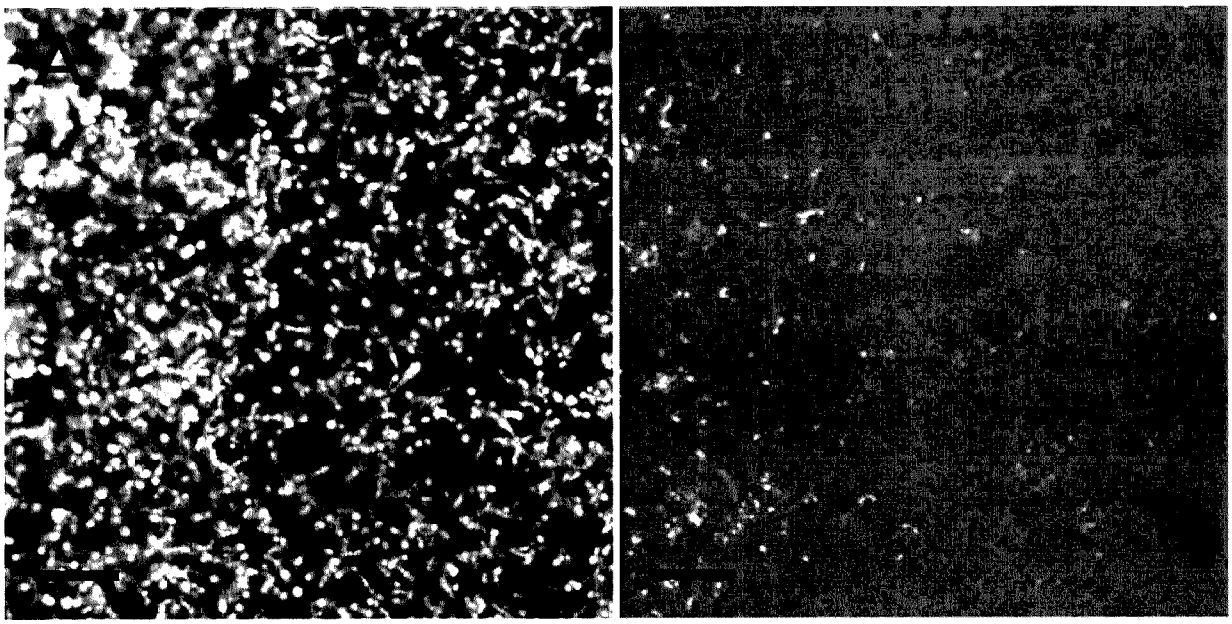


Figure 5

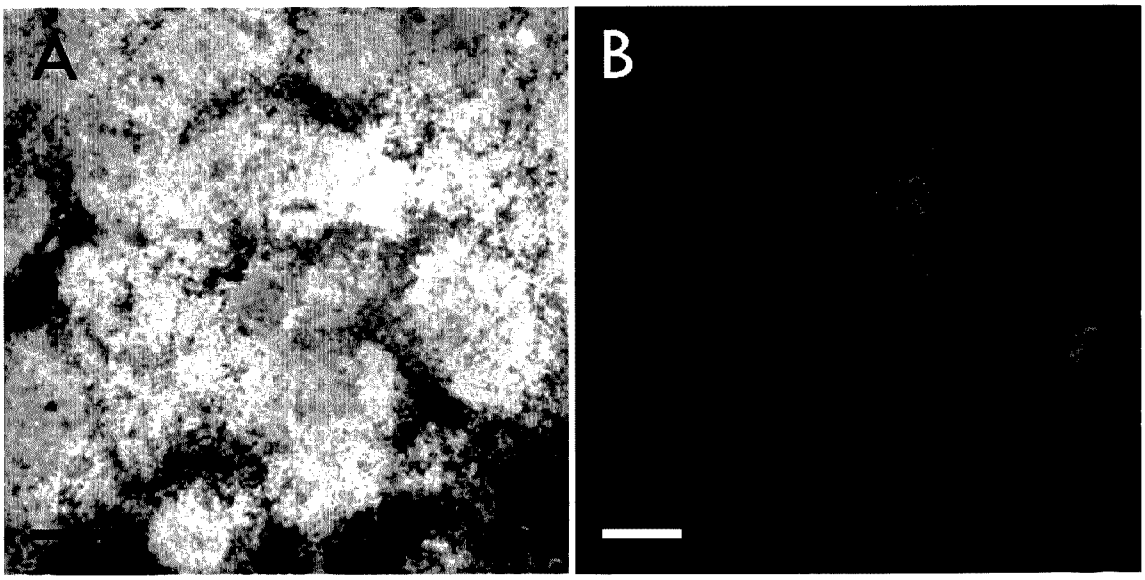


Figure 6

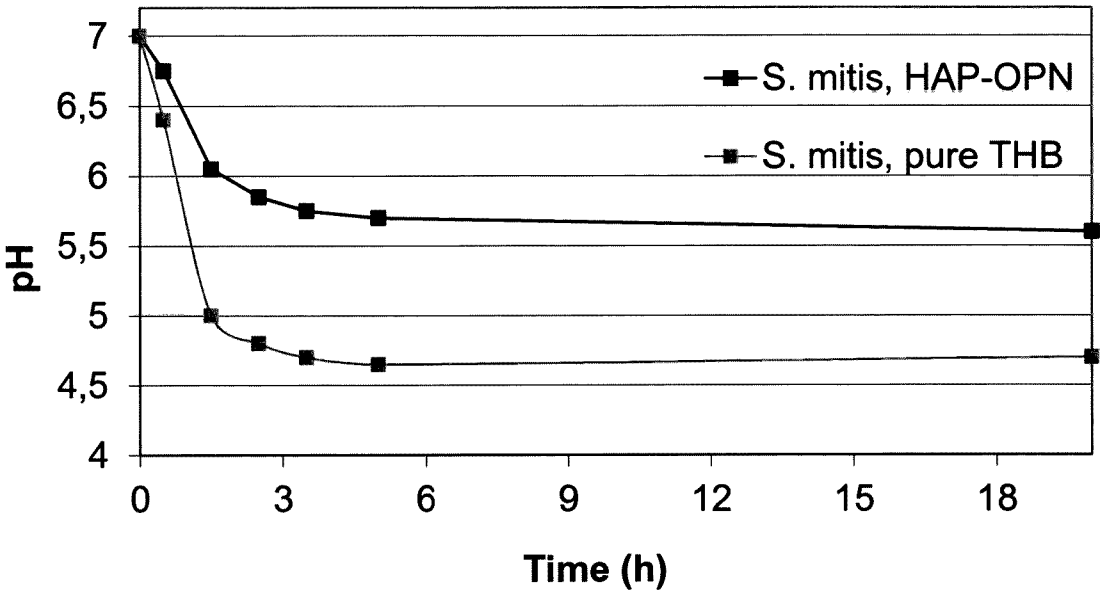

7
6,5
6
5,5
5
4,5
4
pH
0
3
6
9
12
15
18
Time (h)
S. mitis, HAP-OPN
S. mitis, pure THB

Fig. 7a

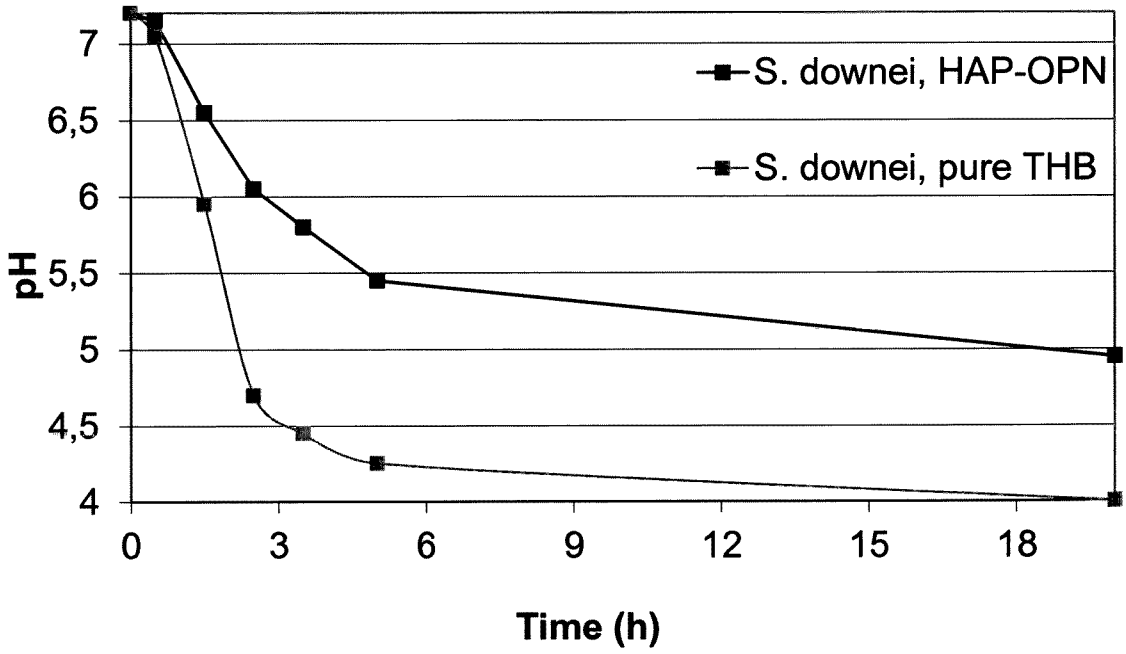

7
6,5
6
5,5
5
4,5
4
pH
0
3
6
9
12
15
18
Time (h)
S. downei, HAP-OPN
S. downei, pure THB

Fig. 7b

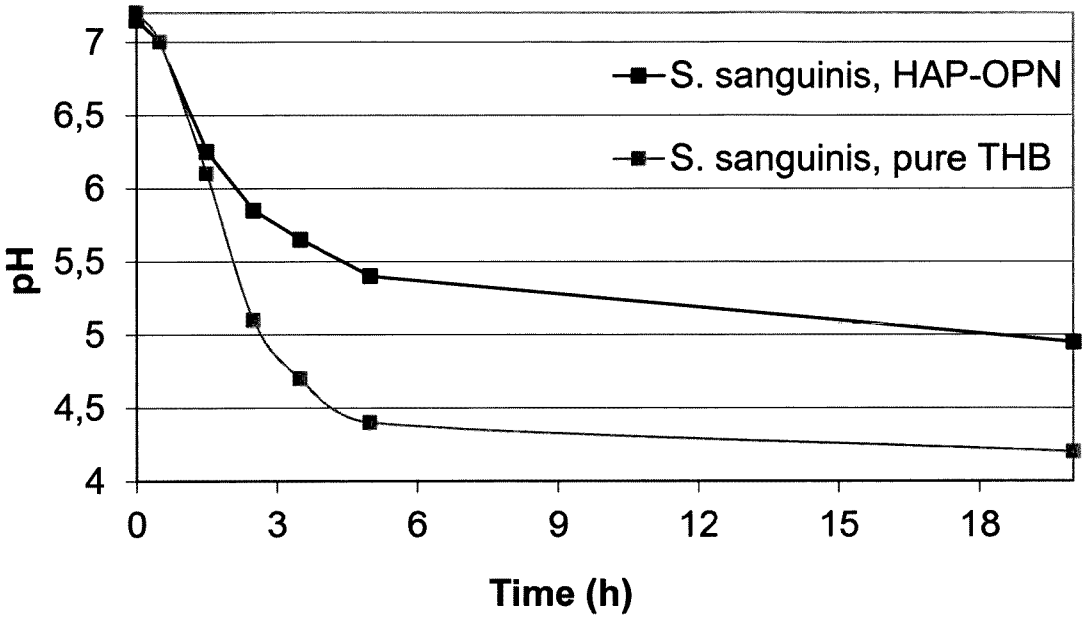

7
6,5
6
5,5
5
4,5
4
pH
0
3
6
9
12
15
18
Time (h)
S. sanguinis, HAP-OPN
S. sanguinis, pure THB

Fig. 7c

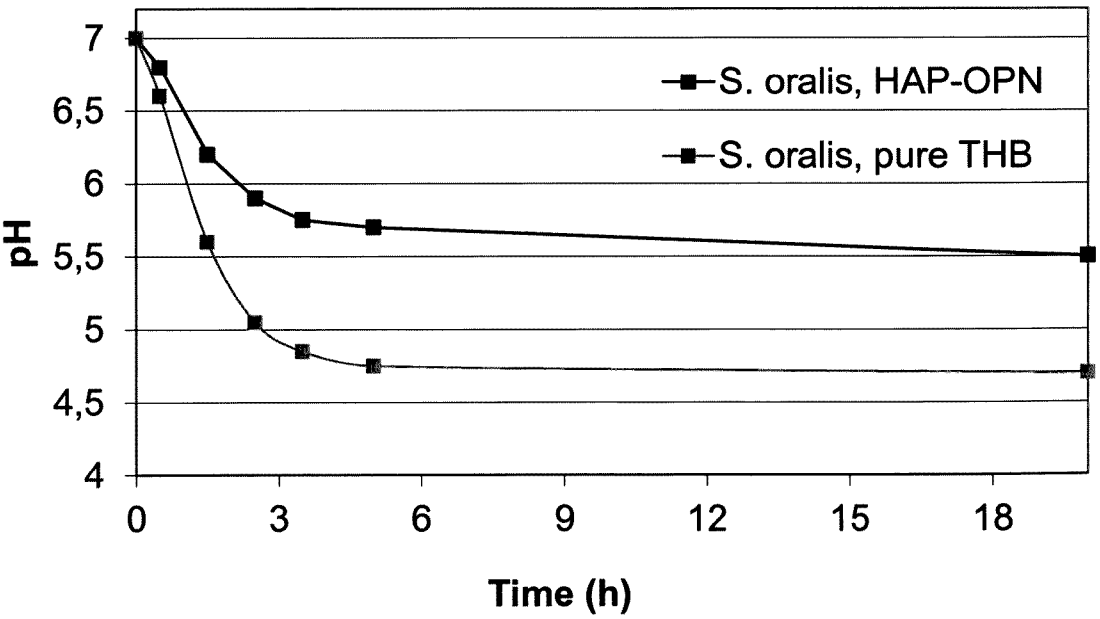

7
6,5
6
5,5
5
4,5
4
pH
0
3
6
9
12
15
18
Time (h)
S. oralis, HAP-OPN
S. oralis, pure THB

Fig. 7d

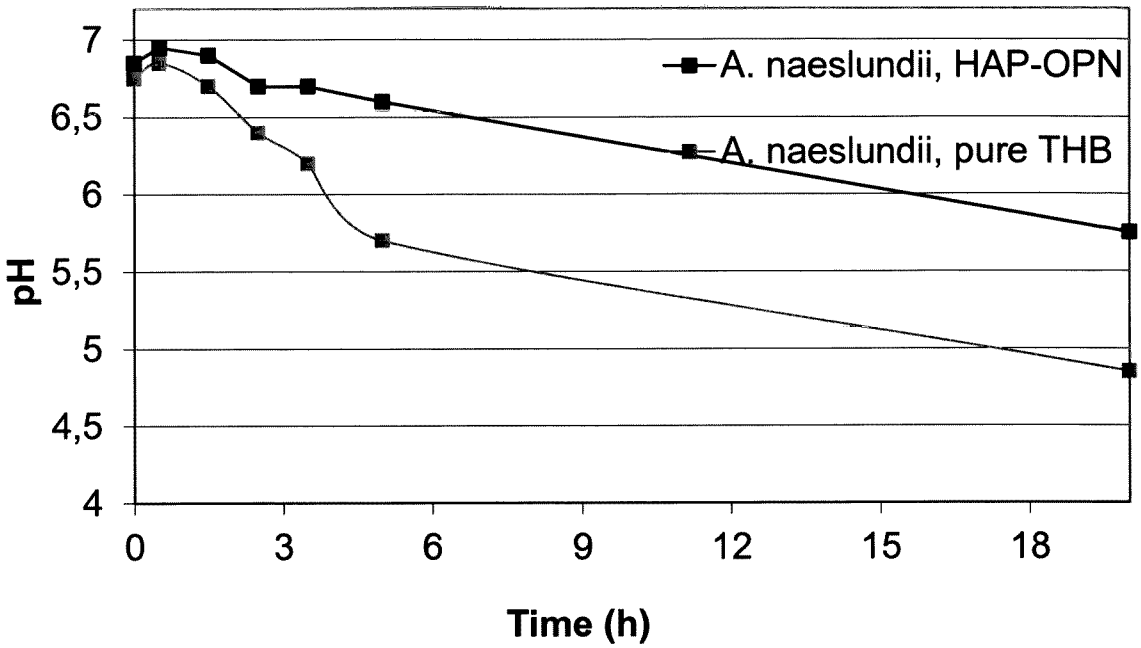
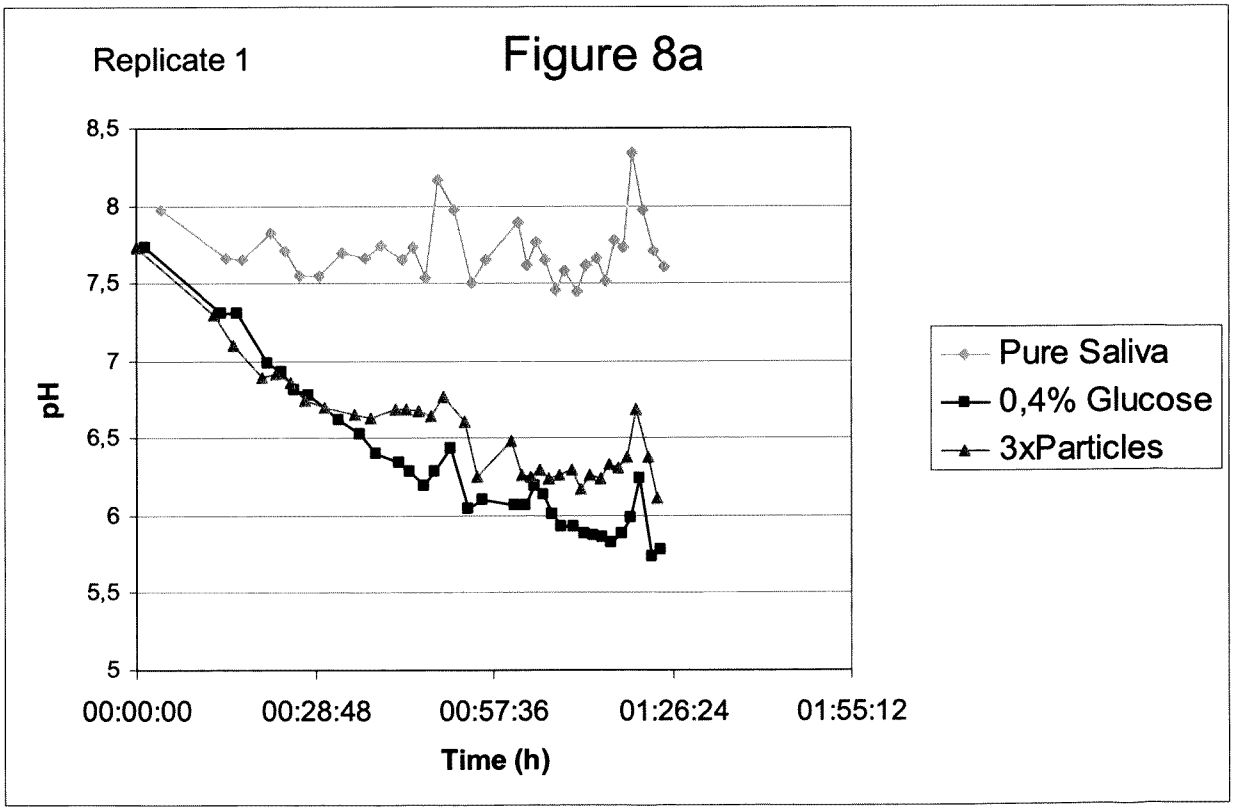
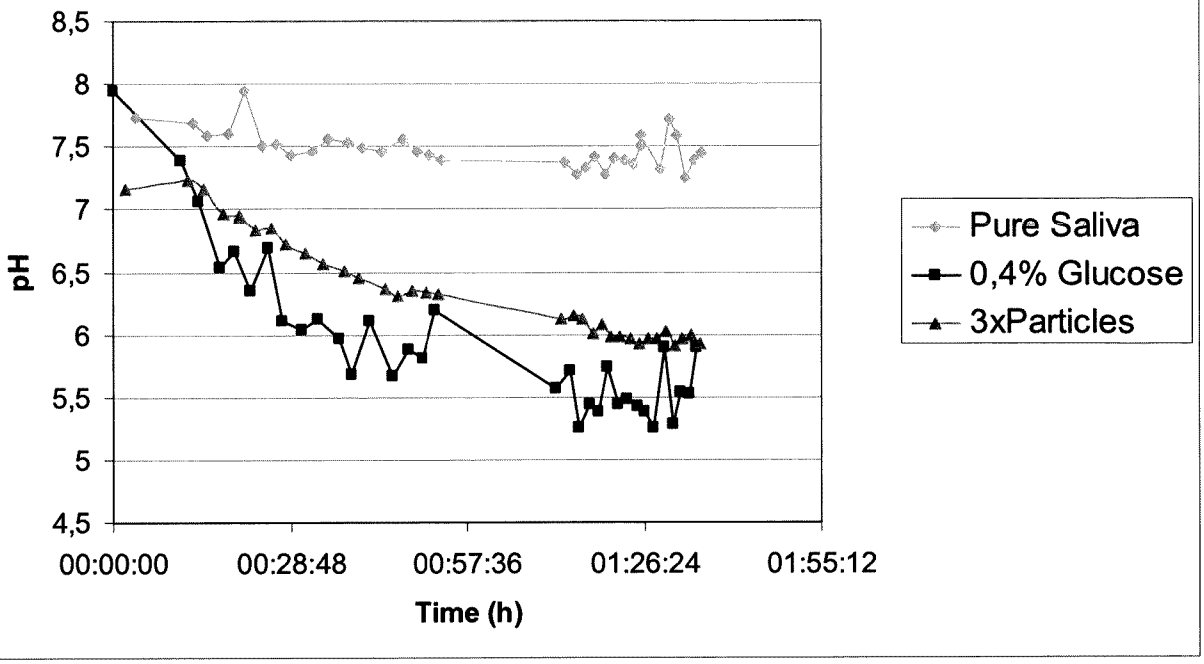

7
6,5
6
5,5
5
4,5
4
pH
0
3
6
9
12
15
18
Time (h)
A. naeslundii, HAP-OPN
A. naeslundii, pure THB

Figure 7e


Replicate 1
Figure 8a
8,5
8
7,5
7
6,5
6
5,5
5
pH
00:00:00
00:28:48
00:57:36
01:26:24
01:55:12
Time (h)
Pure Saliva
0,4% Glucose
3xParticles

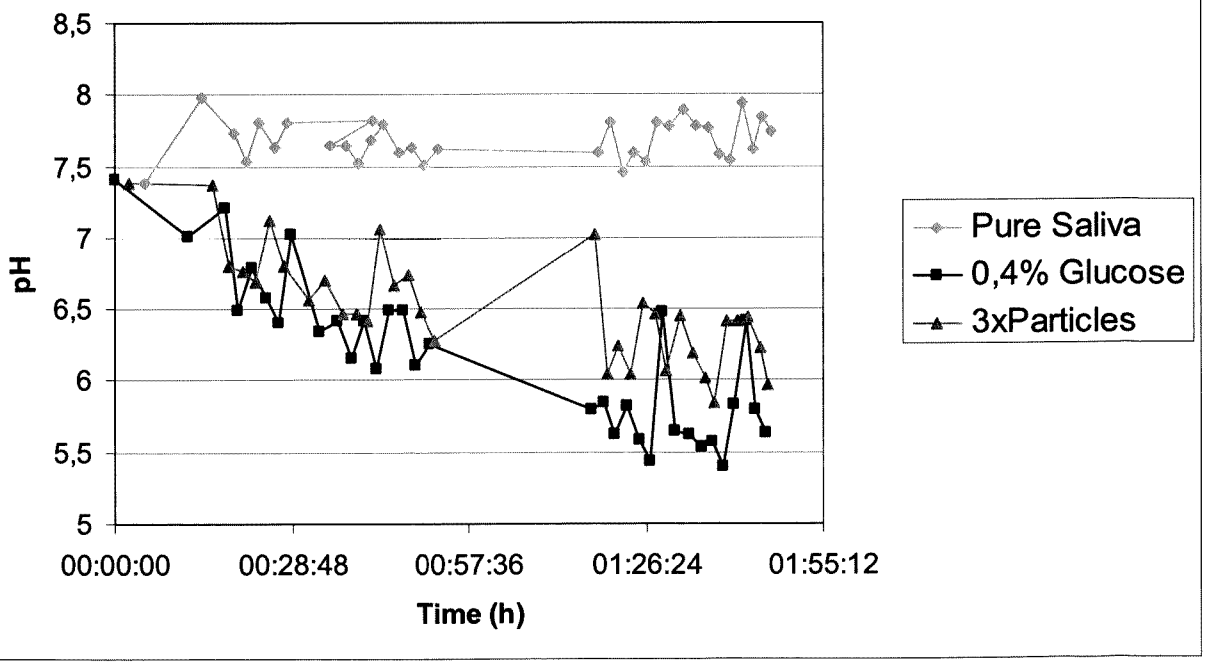
Replicate 2

Figure 8b



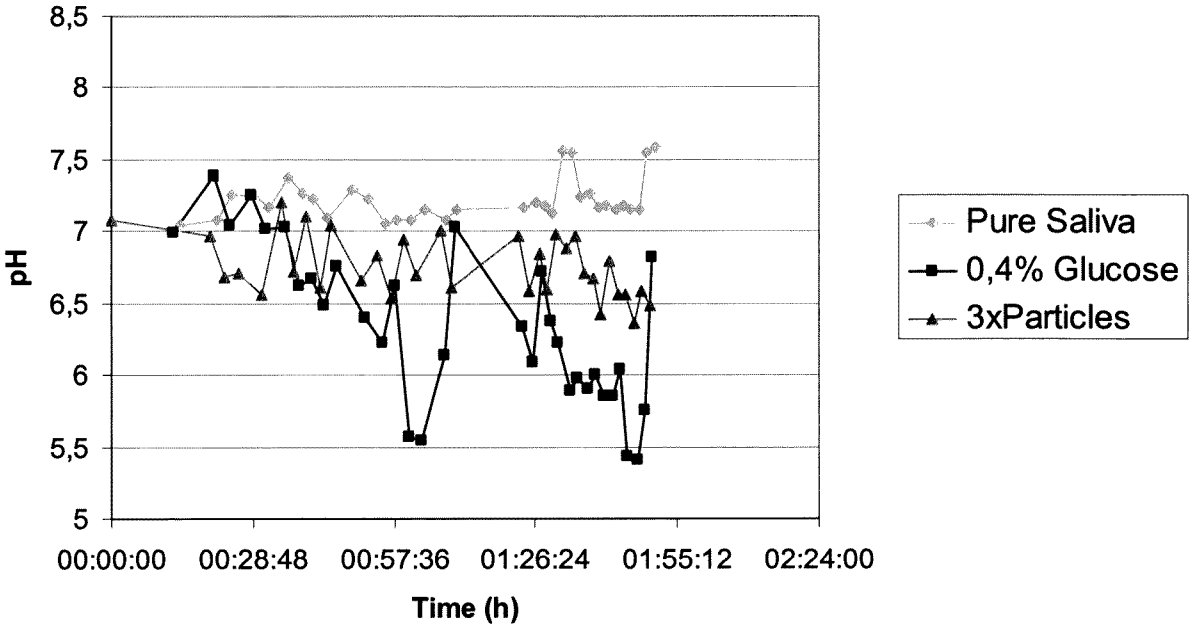
Replicate 3

Figure 8c



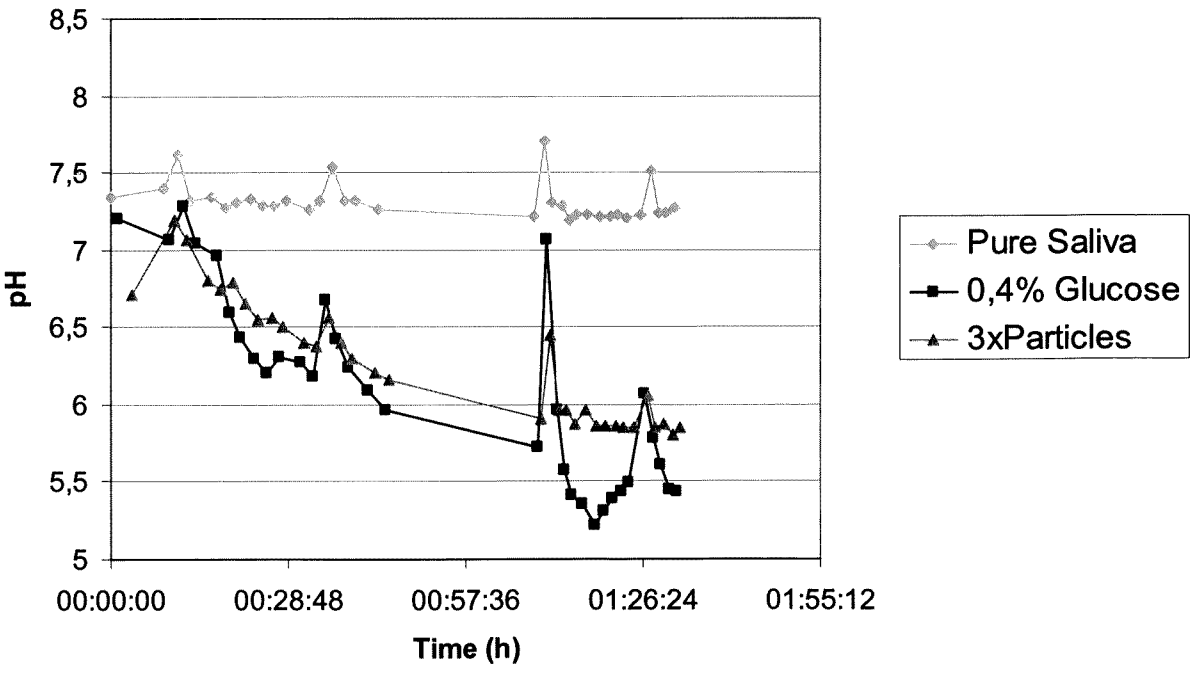
Replicate 4

Figure 8d



Replicate 5

Figure 8e



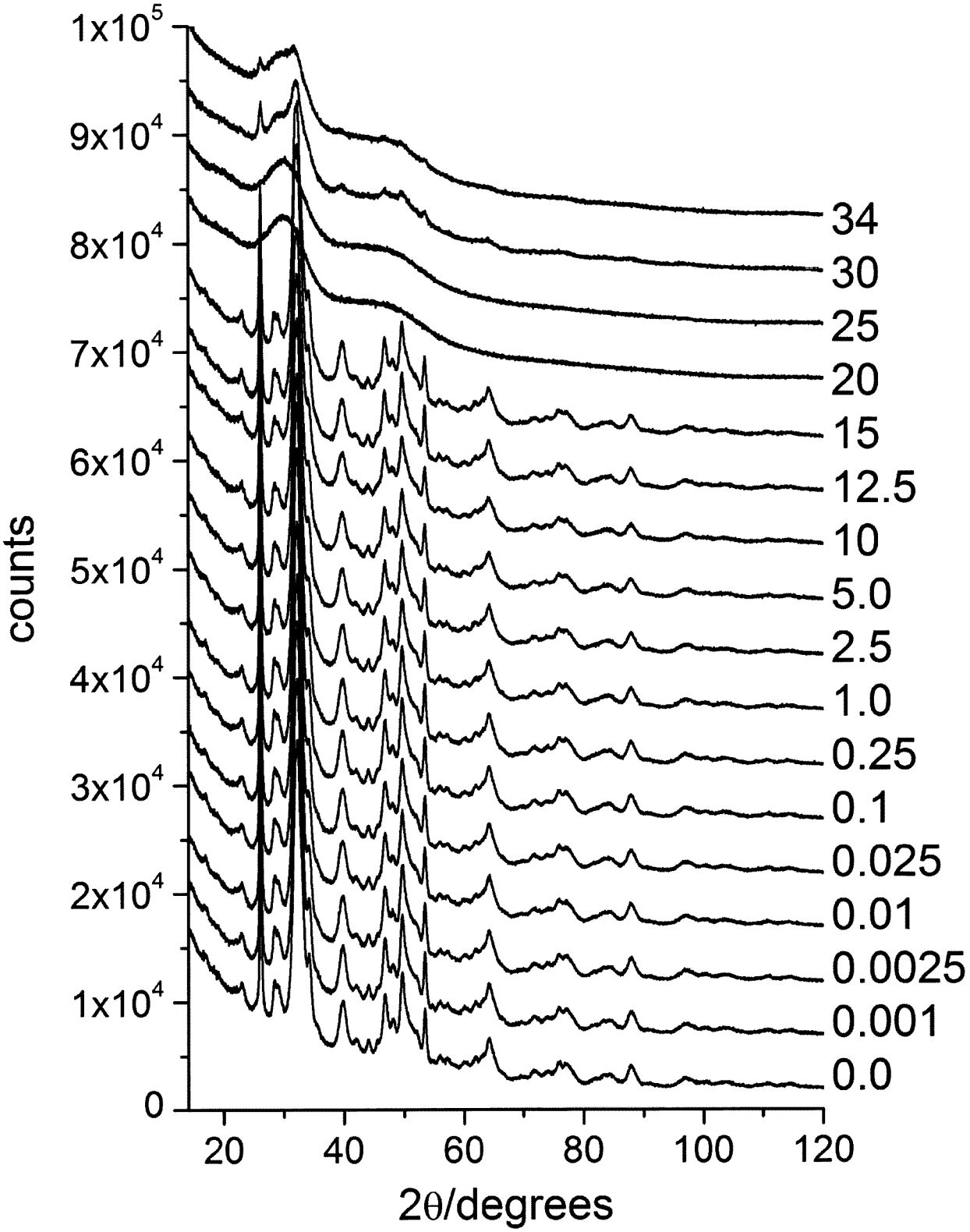

1x10^5
9x10^4
8x10^4
7x10^4
6x10^4
5x10^4
4x10^4
3x10^4
2x10^4
1x10^4
0
counts
20
40
60
80
100
120
2θ/degrees
34
30
25
20
15
12.5
10
5.0
2.5
1.0
0.25
0.1
0.025
0.01
0.0025
0.001
0.0

Figure 9

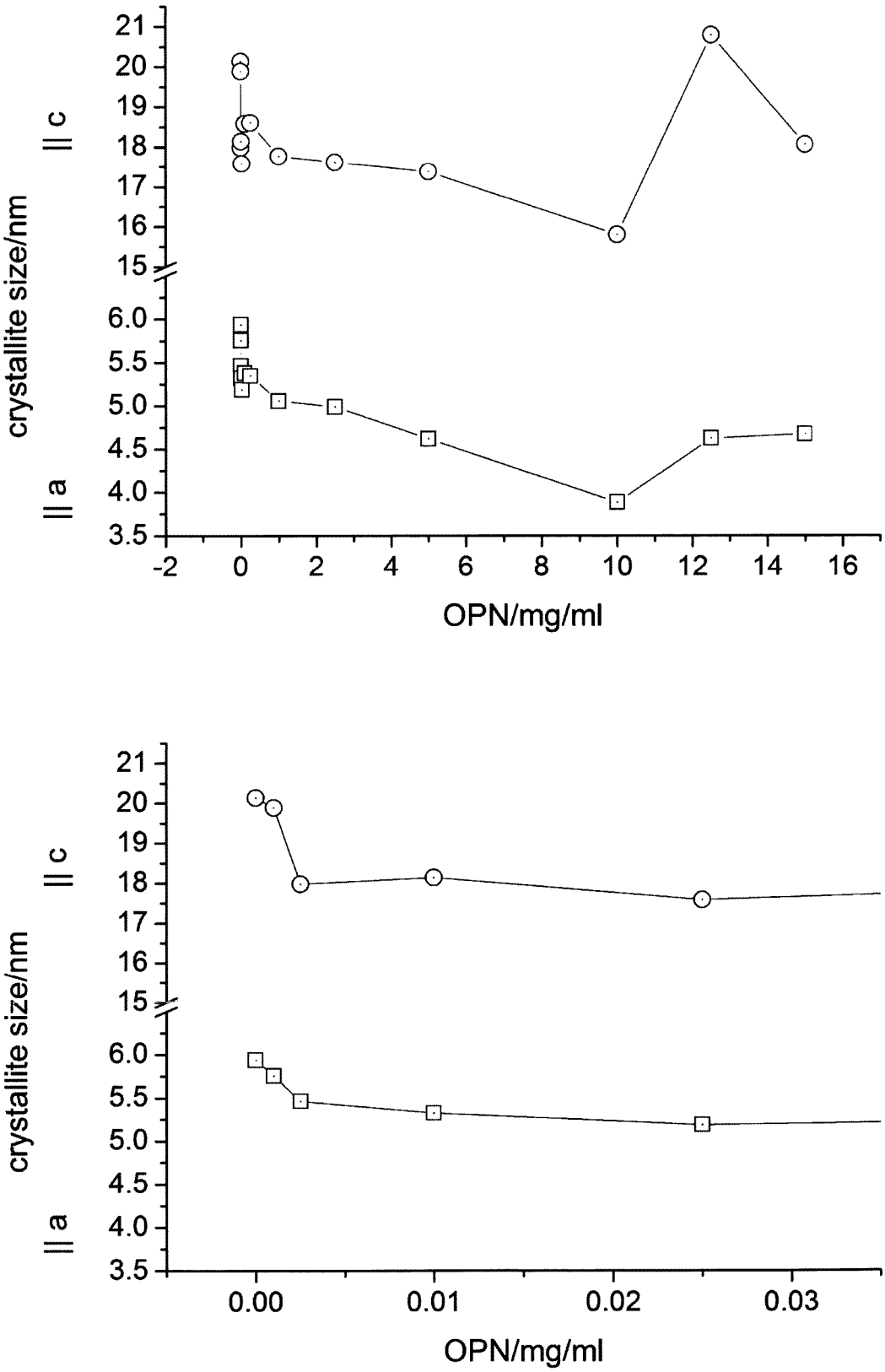

crystallite size/nm
|| c
|| a
21
20
19
18
17
16
15
6.0
5.5
5.0
4.5
4.0
3.5
-2
0
2
4
6
8
10
12
14
16
OPN/mg/ml
0.00
0.01
0.02
0.03

Figure 10

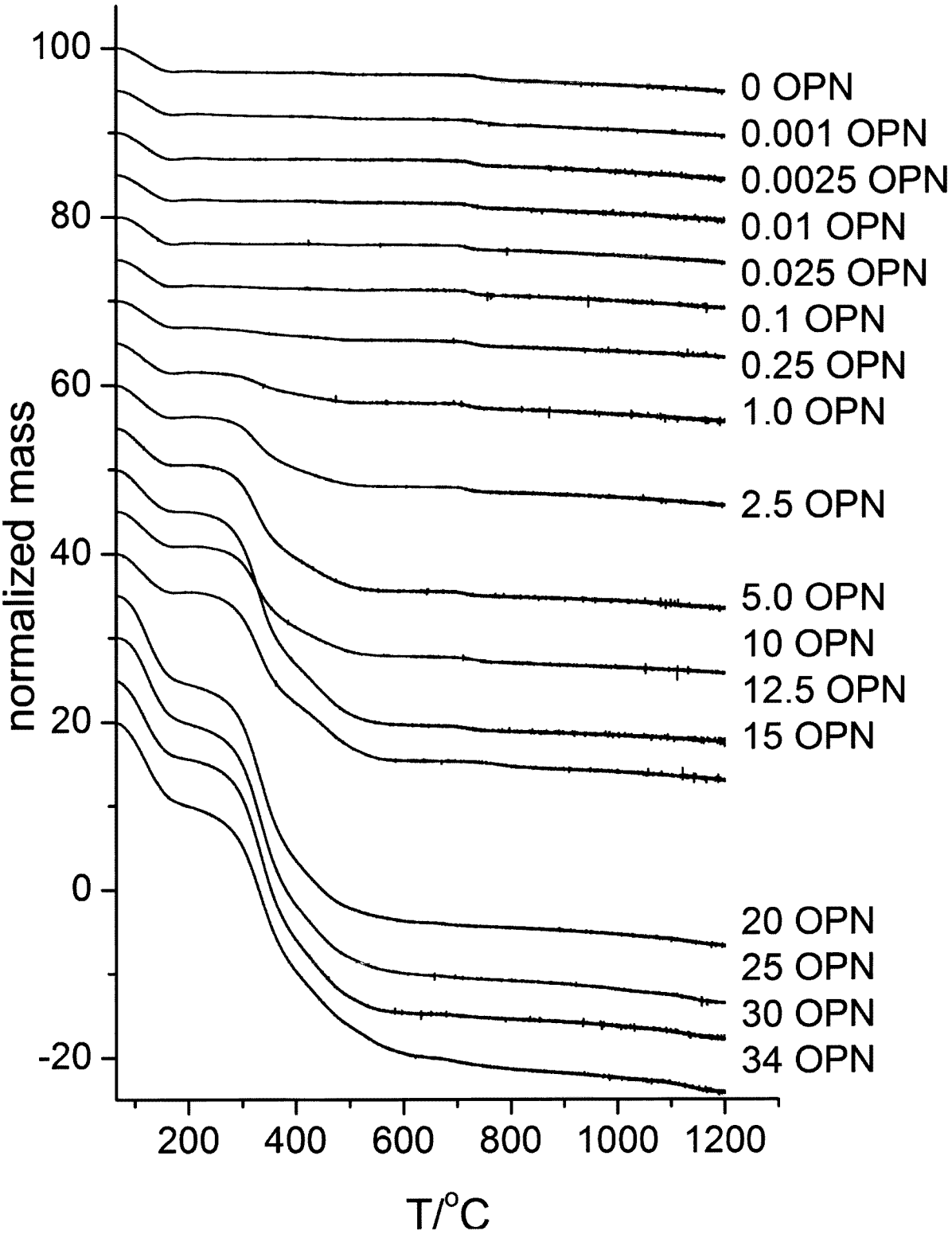

100
80
60
40
20
0
-20
normalized mass
200
400
600
800
1000
1200
T/°C
0 OPN
0.001 OPN
0.0025 OPN
0.01 OPN
0.025 OPN
0.1 OPN
0.25 OPN
1.0 OPN
2.5 OPN
5.0 OPN
10 OPN
12.5 OPN
15 OPN
20 OPN
25 OPN
30 OPN
34 OPN

Figure 11

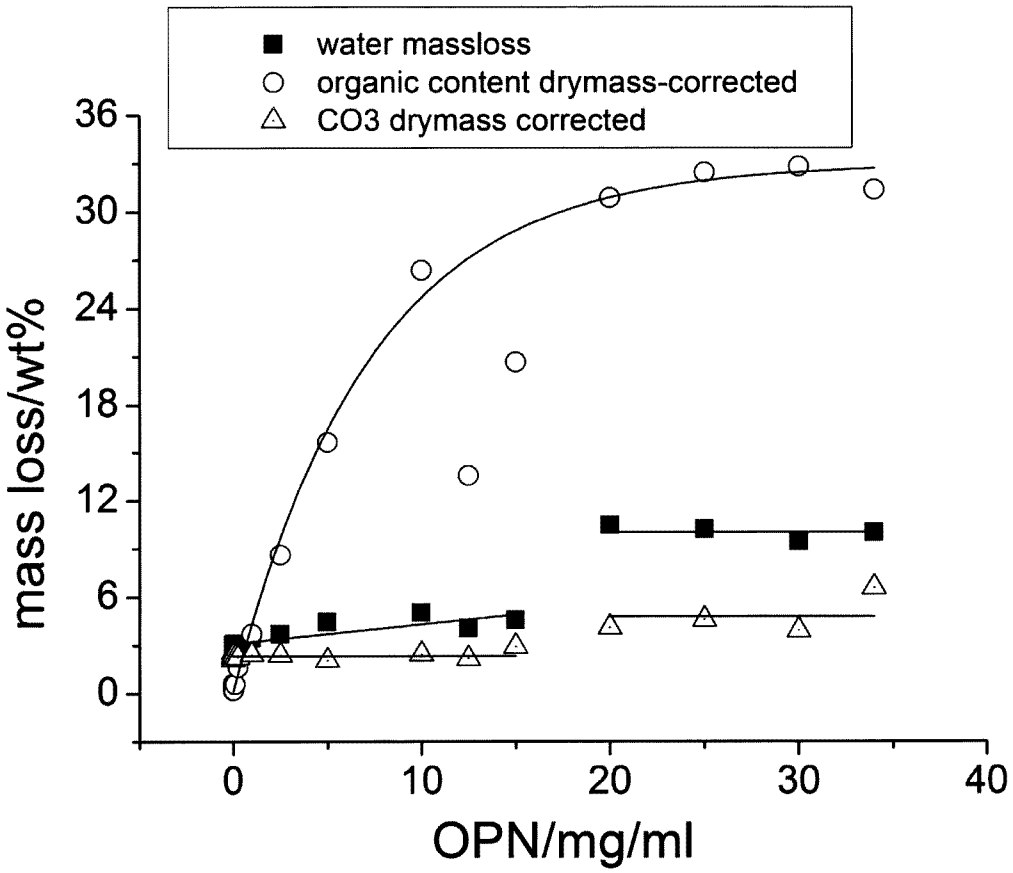

water massloss
organic content drymass-corrected
CO3 drymass corrected
mass loss/wt%
36
30
24
18
12
6
0
0
10
20
30
40
OPN/mg/ml

Figure 12

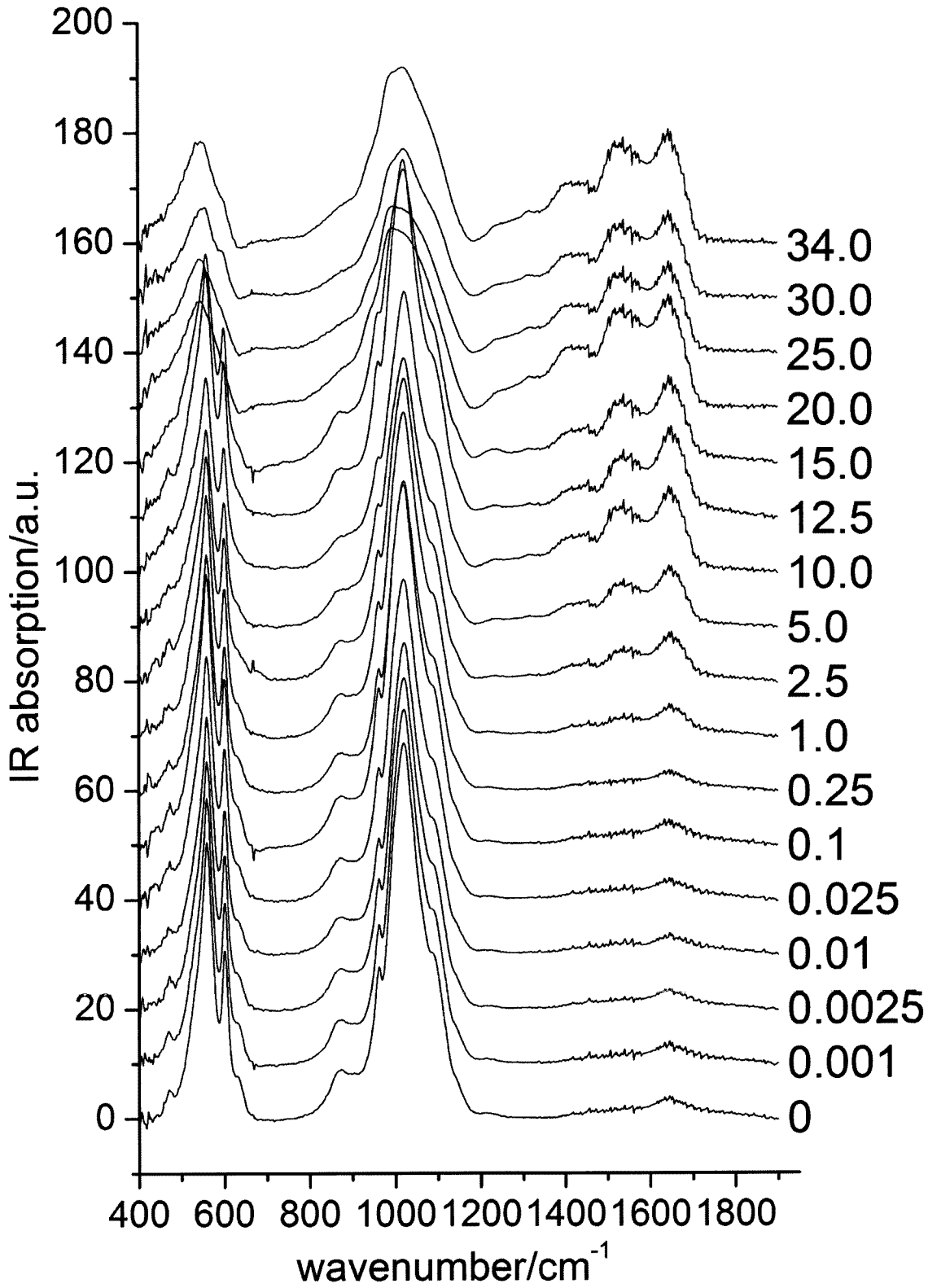

IR absorption/a.u.
200
180
160
140
120
100
80
60
40
20
0
400 600 800 1000 1200 1400 1600 1800
wavenumber/cm-1
34.0
30.0
25.0
20.0
15.0
12.5
10.0
5.0
2.5
1.0
0.25
0.1
0.025
0.01
0.0025
0.001
0

Figure 13

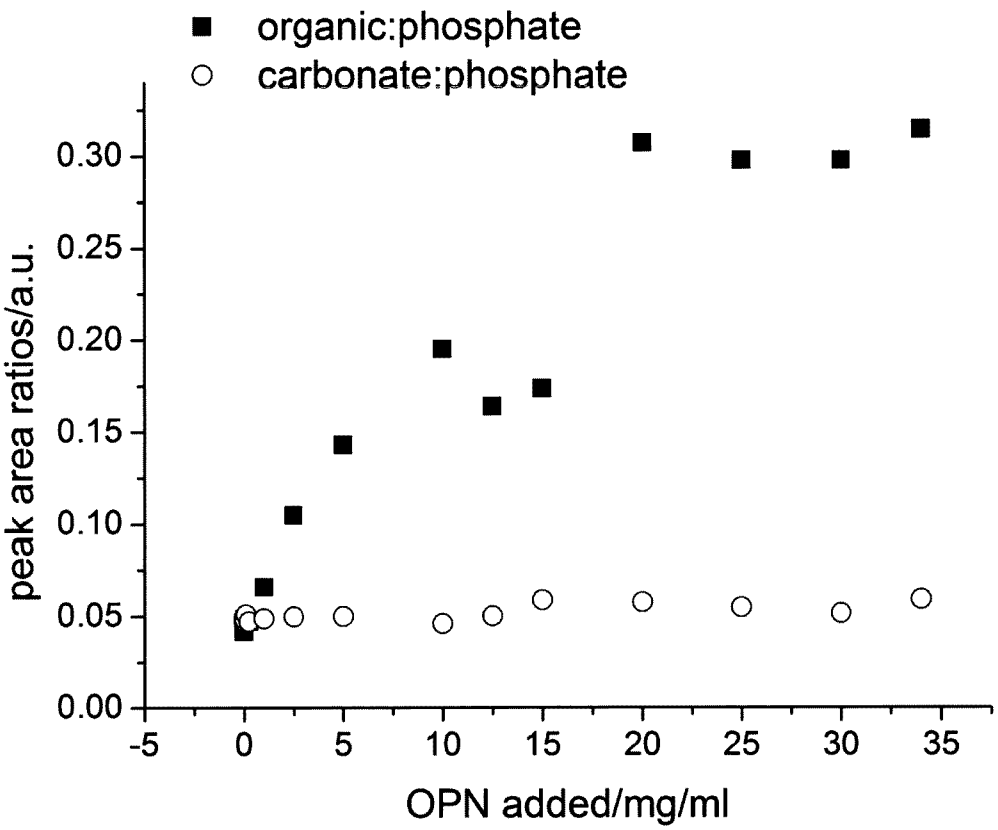

organic:phosphate
carbonate:phosphate
peak area ratios/a.u.
0.00
0.05
0.10
0.15
0.20
0.25
0.30
-5
0
5
10
15
20
25
30
35
OPN added/mg/ml

Figure 14

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/056598

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/19 A61K33/42 A61P31/04
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)
A61K

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01/49741 A2 (ARLA FOODS AMBA [DK]; SOERENSEN ESBEN SKIPPER [DK]; OSTERSEN STEEN [DK) 12 July 2001 (2001-07-12)	1
Y	page 4, line 25 - page 5, line 13	1-25

X	JENSEN T ET AL: "Osteopontin functionalization of hydroxyapatite nanoparticles in a PDLLA matrix promotes bone formation", JOURNAL OF BIOMEDICAL MATERIALS RESEARCH, vol. 99A, no. 1, October 2011 (2011-10), pages 94-101, XP002681784,	1
Y	abstract; paragraph entitled "Coating procedure" on pages 95 and 96	1-25

	-/--	

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"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
8 July 2013	16/07/2013

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	Borst, Markus

1

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

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HOLT CARL ET AL: "Role of calcium phosphate nanoclusters in the control of calcification", FEBS JOURNAL, BLACKWELL PUBLISHING, LONDON, GB, vol. 276, no. 8, 1 April 2009 (2009-04-01) , pages 2308-2323, XP002604801, ISSN: 1742-464X, DOI: 10.1111/J.1742-4658.2009.06958.X abstract; paragraph entitled "Formation of OPN 1-149 nanoclusters" on page 2311; paragraph entitled "Preparation of CPNs" on page 2319	1-25

Y	WO 2009/010071 A1 (UNIV AARHUS [DK]; KJEMS JOERGEN [DK]; HOWARD KENNETH ALAN [DK]; BESENB) 22 January 2009 (2009-01-22) page 3, line 4-21; page 6, line 18-31	1-25

Y	WO 2005/053628 A1 (ARLA FOODS AMBA [DK]; BURLING HANS [SE]; SOERENSEN ESBEN SKIPPER [DK];) 16 June 2005 (2005-06-16) cited in the application page 3, line 14-27	1-25

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2013/056598

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0149741 A2	12-07-2001	AR 027507 A1	02-04-2003
		AT 324378 T	15-05-2006
		AU 784322 B2	09-03-2006
		CA 2396519 A1	12-07-2001
		DE 60119077 T2	07-09-2006
		DK 1244702 T3	28-08-2006
		EP 1244702 A2	02-10-2002
		ES 2258520 T3	01-09-2006
		JP 4615173 B2	19-01-2011
		JP 2003519239 A	17-06-2003
		NO 20023057 A	24-06-2002
		NZ 519492 A	28-03-2003
		PT 1244702 E	31-08-2006
		US 2003149249 A1	07-08-2003
		WO 0149741 A2	12-07-2001
WO 2009010071 A1	22-01-2009	CA 2730841 A1	22-01-2009
		EP 2175841 A1	21-04-2010
		US 2010267139 A1	21-10-2010
		WO 2009010071 A1	22-01-2009
WO 2005053628 A1	16-06-2005	AT 385190 T	15-02-2008
		AU 2004294673 A1	16-06-2005
		BR PI0417098 A	13-03-2007
		CA 2547654 A1	16-06-2005
		CN 1889922 A	03-01-2007
		DE 602004011629 T2	29-01-2009
		DK 1689350 T3	02-06-2008
		EP 1689350 A1	16-08-2006
		ES 2298843 T3	16-05-2008
		JP 5160091 B2	13-03-2013
		JP 2007512379 A	17-05-2007
		JP 2012211195 A	01-11-2012
		KR 20060123235 A	01-12-2006
		NZ 547431 A	26-03-2010
		PT 1689350 E	20-03-2008
		RU 2385162 C2	27-03-2010
		US 2005207996 A1	22-09-2005
		US 2009202449 A1	13-08-2009
		WO 2005053628 A1	16-06-2005