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(54) Title: HYDROPHILIC NONWOVEN NANOFIBERS MEMBRANE FOR PROMOTING BONE REGENERATION

(57) Abstract: The invention relates to a hydrophilic nonwoven nanofiber membrane based on acrylate and methacrylate copolymers and its process of preparation. Furthermore, the invention refers to its hydrolysed form further functionalised with a divalent cation selected from Zn^{+2} , Ca^{+2} , Mg^{+2} and Sr^{+2} , an antibacterial agent and any of the combinations thereof. Moreover the invention refers to a non-resorbable membrane for promoting bone regeneration and a non-resorbable periodontal membrane comprising said hydrophilic nonwoven nanofiber membrane, its hydrolysed form or its hydrolysed form further functionalised with a divalent cation selected from Zn^{+2} , Ca^{+2} , Mg^{+2} and Sr^{+2} , an antibacterial agent and any of the combinations thereof.



DESCRIPTION

HYDROPHILIC NONWOVEN NANOFIBERS MEMBRANE FOR PROMOTING BONE REGENERATION

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The invention relates to a hydrophilic nonwoven nanofiber membrane based on acrylate and methacrylate copolymers and its process of preparation. Furthermore, the invention refers to its hydrolysed form further functionalised with a divalent cation selected from Zn^{+2} , Ca^{+2} , Mg^{+2} and Sr^{+2} , an antibacterial agent and any of the combinations thereof. Moreover, the invention refers to a non-resorbable membrane for promoting bone regeneration and a non-resorbable periodontal membrane comprising said hydrophilic nonwoven nanofiber membrane, its hydrolysed form or its hydrolysed form further functionalised with a divalent cation selected from Zn^{+2} , Ca^{+2} , Mg^{+2} and Sr^{+2} , an antibacterial agent and any of the combinations thereof.

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STATE OF ART

The use of dental implants has become a widespread and predictable treatment modality for the restoration of missing teeth and various edentulous cases. It is clear that the use of a regenerative technique with dental implant placement is an important step which assists the process of bone regeneration. As the clinical success of implant therapy is based on osseointegration, defined as the direct anchorage of the implant in the bone tissue without the interposition of fibrous tissue, considerable research has been conducted to promote bone growth.

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The basic principle of Guided Bone Regeneration (GBR) involves the placement of mechanical barriers to protect blood clots and to isolate the bone defect from the surrounding connective tissue, thus providing bone-forming cells with access to a secluded space intended for bone regeneration. GBR has, in many cases, an unpredictable clinical outcome and remains a challenge. Successful bone regeneration requires: *I)* primary closure of the wound to promote undisturbed and uninterrupted healing, *II)* *angiogenesis* to provide necessary blood supply and undifferentiated *mesenchymal* cells, *III)* space creation and maintenance to facilitate space for bone in-growth and *IV)* stability of the wound to induce blood clot formation and allow uneventful healing. It is usually obtained by utilization of barrier-membranes that are placed after surgery. There are two types of membranes based on the characteristics of resorbability.

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The use of resorbable tissue-engineered matrices to induce bone formation, when additional support is needed, is not always successful. A major limitation of resorbable materials is the inability to exert spatiotemporal control over the wound-healing process. Most of the employed resorbable membranes (e.g. collagen, polylactide-co-glycolide, polycaprolactone) and bone graft substitutes (e.g. hydroxyapatite -HAp- and other calcium phosphates) show a relatively fast rate of biodegradation. It should be taken into account that the healing period of the alveolar bone, after periodontal regeneration or after extraction usually needs 6 to 12 months. Currently, employed resorbable materials may be disadvantageous, as dissolution behaviors are not as long-lasting as required. Moreover, some degradation products from these resorbable materials have low pH, they may not be cytocompatible and could also alter the remineralization processes [Ivanovski S, Vaquette C, Gronthos S, Hutmacher DW, Bartold PM (2014) Multiphasic Scaffolds for Periodontal Tissue Engineering. J Dent Res 93(12):1212-1221] [Shimauchi H, Nemoto E, Ishihata H, Shimomura M (2013) Possible functional scaffolds for periodontal regeneration. Japan Dent Sci Rev 49:118-130].

Although much research is being conducted on resorbable periodontal membranes, non-resorbable synthetic membranes of polytetrafluoroethylene (PTFE), still represent the gold-standard for clinicians, due to the higher predictability of their effects when compared to resorbable membranes. However, PTFE possess important disadvantages: *I*) low adhesiveness for cells, *II*) total absence of the capability of connecting to the bone tissue and providing osseointegration, without formation of a connective tissue interlayer; then a second surgery is required to remove the non-integrated membrane, and finally *III*) lack of antibacterial properties, being infections frequently observed [Sam G, Pillai BRM (2014) Evolution of Barrier Membranes in Periodontal Regeneration-Are the third Generation Membranes J of Clin Diagn Res 8: 14-17]. Therefore, a successful membrane for GBR should resemble the morphology of natural bone. Natural bone is a hybrid of inorganic-organic tissue composed of hydroxyapatite nanocrystals and collagen fibers (with diameters ranging from 50 to 500nm) assembled into a porous mesh, with interconnected pores. Bone is nanostructured, so nanosized materials should be the best choice for bone substitutes.

For the reasons stated above, it is needed to develop new membranes suitable for bone regeneration.

DESCRIPTION OF THE INVENTION

5 A first aspect of the present invention relates to a hydrophilic nonwoven nanofibers membrane (herein “the membrane of the invention”) characterised in that it comprises a blend of

- a first copolymer of $(MA)_3\text{-co-}(HEA)_2$ with statistical topology in a weight percent between 35% and 65%; and
 - a second copolymer of $(MMA)_1\text{-co-}(HEMA)_1$ in weight percent between 35% and 65%.
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The term “hydrophilic nonwoven nanofibers membrane” refers to a membrane formed by long fibers having a diameter of between 150nm and 400nm. Said membranes are nonwoven, this means that they are like a felt, which are neither woven nor knitted: they are made from long fibers (continuous long), bonded together by chemical, mechanical, heat or solvent treatment, and have a hydrophilic character.

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Ideally each needle produces a single fiber that is wound on the drum from the beginning to the end of the electrospinning process (kilometric). The reality is that fibers are cut intermittently along the electrospinning process.

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The term “copolymer with statistical topology” refers to statistical copolymers, that is to say, a copolymer in which the distribution of the monomers in the chain is random since all the monomers present in the solution have the same affinity/probability to react both with monomers of the same chemical nature (with themselves) and with monomers of a different chemical nature.

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In a preferred embodiment of the membrane of the invention it comprises a blend of

- a first copolymer of $(MA)_3\text{-co-}(HEA)_2$ with statistical topology in a 50% by weight; and
- a second copolymer of $(MMA)_1\text{-co-}(HEMA)_1$ in a 50% by weight.

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Said membrane exhibits abrasion resistance, flexibility, elasticity, stress resistance, and thus it can be easily manipulated: can be cut, bend and twist.

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One of the most important parameter in the electrospinning process is the molecular weight of the polymer. Higher molecular weight is generally preferred as there will be greater chains entanglement which facilitates the formation of fibers during spinning. In contrast, lower molecular weight may break up into droplets forming beads or beads combined with short fibers, resulting in heterogeneous materials with unwanted physical properties: irregular surface, low specific surface, low resistance to abrasion, and stress, loss of elasticity.

In another preferred embodiment of the membrane of the invention, the first copolymer of (MA)₃-co-(HEA)₂ has molecular weight between 50000 Da and 3·10⁶ Da, preferably above 80000 Da. More preferably, the first copolymer of (MA)₃-co-(HEA)₂ has molecular weight between 1·10⁶ Da and 3·10⁶ Da.

In another preferred embodiment of the membrane of the invention, the second copolymer of (MMA)₁-co-(HEMA)₁ has molecular weight between 50000 Da and 1·10⁶ Da.

In another preferred embodiment of the membrane of the invention, the membrane further comprises SiO₂ nanoparticles (NPs-SiO₂), and said SiO₂ nanoparticles are

- homogenously dispersed in the membrane, this is, trapped homogeneously in the whole fiber volume forming a solid solution (composite) and/or
- physically adsorbed on the surface of the membrane,

in a weight percent between 0.1% and 60% with respect to the final weight of the membrane.

Silicon dioxide (SiO₂) is able to improve not only bioactivity of materials but also cell adhesion and proliferation on artificial tissues, facilitating osteogenic cells differentiation. SiO₂ is considered to be osteoinductive and a catalyst for bone formation. Therefore, in order to improve the bioactivity of membranes they were doped with SiO₂ nanoparticles.

The SiO₂ nanoparticles (NPs-SiO₂) can be introduced in the membranes by two ways: 1) suspending them in the electrospinning solution, and then carry out the electrospinning process. In this case the NPs-SiO₂ are trapped homogeneously into the whole fiber volume, forming a solid solution (composite);

2) by physical adsorption in the surface of the fibers once the membrane is made: the membrane is soaked with a suspension of NPs-SiO₂, and then the water is evaporated.

5 The option (1) is preferred because NPs-SiO₂ are retained in the fibers more efficiently and its leaching is minimized; by (1) the NPs- SiO₂ can remain in the membrane for longer times than by (2).

10 A second aspect of the present invention relates to a process of preparation of the membrane of the present invention (herein "the process of the invention") that includes the preparation of the copolymers which are electrospun to produce said membrane. The copolymers of the present invention can be prepared by Conventional Free-Radical Polymerization or by Metal-Catalysed Living Radical Polymerization (MC-LRP) such as normal Atom Transfer Radical Polymerization (Normal ATRP), reverse Atom Transfer Radical Polymerization (reverse ATRP) and activator generated by electron transfer atom transfer radical polymerization (AGET ATRP).

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In a preferred embodiment of the process of the invention, the process is characterised in that it comprises the following steps:

- 20 a) synthesis of the first copolymer of (MA)₃-co-(HEA)₂ with statistical topology by Metal-catalysed living radical polymerization using a metal catalytic system;
- b) synthesis of the second copolymer of (MMA)₁-co-(HEMA)₁ by reverse atom transfer radical polymerization using a metal catalytic system;
- 25 c) preparation of a nanofibers membrane comprising a blend, said blend comprising the first copolymer obtained in step (a) and the second copolymer obtained in step (b) by electrospinning, and
- d) heat treatment of the nanofibers membrane obtained in step (c), wherein the heat treatment is applied in the form of hot water at a temperature range between 30 °C and 80 °C, for instance for at least 4 hours, and wherein the nanofibers membrane obtained in step (c) is kept tensioned by means of a
- 30 frame.

Step (a) of the process of the invention refers herein to the synthesis of the first copolymer of (MA)₃-co-(HEA)₂ by Metal-Catalysed Living Radical Polymerization using a metal catalytic system and step (b) refers to the synthesis of the second copolymer of (MMA)₁-co-(HEMA)₁ by Reverse Atom Transfer Radical Polymerization using a metal

35 catalytic system.

5 The term "Metal-catalysed living radical polymerization" refers to polymerization methods based on establishing a rapid dynamic equilibration between a minute amount of growing free radicals and a large majority of the dormant species, in which a low oxidation state metal complex acts as the catalyst.

10 The term "Reverse Atom Transfer Radical Polymerization" refers to the polymerization methods based on establishing a rapid dynamic equilibration between a minute amount of growing free radicals and a large majority of the dormant species, in which a low oxidation state metal complex acts as the catalyst, the dormant species are alkyl halides, and the reaction is initiated by a conventional radical initiator and a Cu^{II} complex.

15 In the present invention the term "metal catalytic system" refers to the catalyst used in the Metal-Catalysed Living Radical Polymerization of step (a) and to the catalyst used in the reverse Atom Transfer Radical Polymerization of step (b). Said metal catalytic system comprises a metal, a ligand and an initiator and uses a particular solvent. Preferably, the metal catalytic system of step (a) and step (b) is a copper amine complex.

20 Preferably, the metal of the metal catalytic system of step (a) and/or step (b) comprises a transition metal or a mixture of transition metals in different oxidation states. More preferably, the metal of the metal catalytic system of step (a) and step (b) is independently selected from the list consisting of Cu, Fe, Co, Ni, Ru, Pt, Rh, Re, Cr and Mo.

25 Preferably, said metal of the metal catalytic system of step (a) and/or step (b) is in a weight percentage comprised between 0.00001% and 0.1%.

30 Preferably, the ligand of the metal catalytic system of step (a) and/or step (b) is a multidentate aliphatic amine which can be linear or branched. More preferably, the ligand of the metal catalytic system of step (a) and step (b) is independently selected from the list consisting of N,N,N',N'',N'''-Pentamethyldiethylenetriamine (PMDETA) Tris(2-pyridylmethyl)amine, Tris[2-(dimethylamino)ethyl]amine, 2,2'-Bipyridyl, N,N,N',N'''-Tetrakis(2-pyridylmethyl)ethylenediamine and 1,1,4,7,10,10-

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Hexamethyltriethylenetetramine. Preferably, said ligand is in a weight percentage between 0.0001% and 0.2%.

It is to mention that the initiator of step (a) and step (b) are preferably different.

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Preferably, the initiator of the metal catalytic system of step (a) is independently selected from the list consisting of Dodecyl 2-bromoisobutyrate, Ethyl α -bromoisobutyrate, Ethyl α -bromoisobutyrate, Octadecyl 2-bromoisobutyrate, Methyl α -bromoisobutyrate, Methyl 3-bromopropionate, tert-Butyl 3-bromopropionate, Ethyl 2-bromopropionate.

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Preferably the initiator of step (b) is independently selected from the list consisting of 1,1'-azobis(cyclohexanecarbonitrile) (ACHN), 2,2'-azobis (2-methylpropionamidine) 2,2'-dihydrochloride (AAPH), 4,4'-azobis(4-cyanovaleric acid) (ACVA), tert-butyl hydroperoxide, cumene hydroperoxide, 2,5-di(tert-butylperoxide)-2,5-dimethyl-3-hexyne, dicumyl peroxide and 2,5-bis(tert-butylperoxide)-2,5-dimethylhexane.

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The weight percentage of the initiator in step (a) and step (b) is between 0.00001% and 0.2%.

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Preferably, the solvent used with the metal catalytic system of step (a) and step (b) is independently selected from the list consisting of acetone, dimethyl formamide, poly(ethylene glycol), dimethyl sulfoxide, 1-4 Dioxane, ethanol, propanol, hexane, water, carbon dioxide, ionic liquid, and a combination thereof.

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The weight percentage of the solvent in step (a) and step (b) is below 90%; preferably the weight percentage of the solvent in step (a) and step (b) is between 40% and 60%.

In another preferred embodiment of the present invention, no solvent is used with the metal catalytic system of step (a) and step (b). In other words, step (a) and step (b) are carried out without solvent since monomers are liquids and miscible to each other.

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In a preferred embodiment of the process of the present invention, the metal catalytic system of step (a) uses $\text{Cu}^0/\text{Cu}^{2+}$ as transition metal, Tris(2-dimethylaminoethyl)amine as ligand, Methyl 2-bromopropionate as initiator, and dimethyl sulfoxide as solvent.

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Step (c) of the process of the invention refers to the preparation of a nanofibers membrane comprising a blend, said blend comprising the first copolymer obtained in step (a) and the second copolymer obtained in step (b) by electrospinning. Preferably, step (c) is performed in the presence of an additive capable of increasing the conductivity of the solution blend/solvent. More preferably, in the presence of hydrochloric acid (HCl), wherein the weight percent of HCl in step (c) is between 0.0001% and 0.2%.

The solvent of step (c) is selected from the list consisting of acetone, dimethyl formamide, poly(ethylene glycol), dimethyl sulfoxide, 1-4 Dioxane, ethanol, propanol, hexane, water, carbon dioxide, ionic liquid, and a combination thereof. More preferably, the solvent of step (c) is dimethyl sulfoxide.

Preferably, the weight percent of the solvent used in step (c) is ranging between 20% and 98%.

Step (d) refers to a heat treatment of the nanofibers membrane obtained in step (c), wherein the heat treatment is applied in the form of hot water at a temperature range between 30°C and 80°C and wherein the nanofibers membrane obtained in step (c) is kept tensioned by means of a frame. The aim of this step (d) is to convert the nanofibers membrane obtained in step (c) from hydrophobic to hydrophilic; a visual transformation of the membrane is observed when wet thermal treatment of step (d) is performed for at least 4 hours. Please note that the membrane lasts hydrophilic for days, even years.

A third aspect of the invention refers to a process of preparation of the hydrophilic nonwoven nanofibers membrane comprising SiO₂ nanoparticles, wherein said SiO₂ nanoparticles are homogenously dispersed in the membrane, characterised in that it comprises all steps of the process of the invention:

- a) synthesis of the first copolymer of (MA)₃-co-(HEA)₂ by Metal-Catalysed Living Radical Polymerization using a metal catalytic system;
- b) synthesis of the second copolymer of (MMA)₁-co-(HEMA)₁ by Reverse Atom Transfer Radical Polymerization using a metal catalytic system;
- c) preparation of a nanofibers membrane comprising a blend, said blend comprising the first copolymer obtained in step (a) and the second copolymer obtained in step (b) by electrospinning, and

d) heat treatment of the nanofibers membrane obtained in step (c), wherein the heat treatment is applied in the form of hot water at a temperature range between 30°C and 80°C, for instance for at least 4 hours, and wherein the nanofibers membrane obtained in step (c) is kept tensioned by means of a frame.

and wherein the blend of step (c) comprises SiO₂ nanoparticles.

Another aspect of the invention refers to a process of preparation of the hydrophilic nonwoven nanofibers membrane comprising SiO₂ nanoparticles, wherein said SiO₂ nanoparticles are physically adsorbed, characterised in that it comprises all steps of the process of the invention:

a) synthesis of the first copolymer of (MA)₃-co-(HEA)₂ by Metal-Catalysed Living Radical Polymerization using a metal catalytic system;

b) synthesis of the second copolymer of (MMA)₁-co-(HEMA)₁ by reverse Atom transfer radical polymerization using a metal catalytic system;

c) preparation of a nanofibers membrane comprising a blend, said blend comprising the first copolymer obtained in step (a) and the second copolymer obtained in step (b) and optionally comprises SiO₂ nanoparticles, by electrospinning, and

d) heat treatment of the nanofibers membrane obtained in step (c), wherein the heat treatment is applied in the form of hot water at a temperature range between 30°C and 80°C, for instance for at least 4 hours, and wherein the nanofibers membrane obtained in step (c) is kept tensioned by means of a frame.

and a further step (e) of soaking the membrane obtained in step (d) in a suspension of NPs- SiO₂ and evaporate the solvent.

Another aspect of the invention refers to hydrolysed hydrophilic nonwoven nanofibers membrane (herein the hydrolysed membrane of the present invention) characterised in that it comprises the hydrophilic nonwoven nanofibers membrane comprising carboxyl groups, wherein the concentration of carboxyl groups in the membrane is ranging between 20 µmol/g of the membrane and 3000 µmol/g of the membrane.

The term “hydrolysed hydrophilic nonwoven nanofibers membrane” refers herein to the hydrophilic nonwoven nanofibers membrane mentioned above which has been partially hydrolysed and now comprises carboxyl groups (COOH) and dried afterwards at room

temperature (18-28°C). The number of accessible COOH groups in the membrane is ranging between 20 µmol/g of the membrane and 3000 µmol/g of the membrane. It was found that a hydrolysis time longer than 1 hour, produced a high rigidity in the membranes, making them fragile and brittle.

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In a preferred embodiment of the hydrolysed membrane of the present invention, said membrane is functionalised with a divalent cation selected from Zn^{+2} , Ca^{+2} , Mg^{+2} and Sr^{+2} , an antibacterial agent and/or any of the combinations thereof. More preferably, the hydrophilic nonwoven nanofibers membrane is functionalised with Zn^{+2} , Ca^{+2} and doxycycline.

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The functionalization of the hydrolysed membrane of the present invention with a divalent cation selected from Zn^{+2} , Ca^{+2} , Mg^{+2} and Sr^{+2} comprises a step of soaking the hydrolysed with a solution of a divalent cation selected from Zn^{+2} , Ca^{+2} , Mg^{+2} and Sr^{+2} , and a step of drying at room temperature (18-28°C).

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The functionalization of the hydrolysed membrane of the present invention with an antibacterial agent, more preferably doxycycline, comprises a step of soaking the hydrolysed with a solution of an antibacterial agent and a step of drying at room temperature (18-28°C).

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According to the accessible COOH groups in the hydrolysed hydrophilic nonwoven nanofibers membrane, the concentration of Ca^{2+} and Zn^{2+} loaded in the membrane as $(\text{COO}^-)_2$ is ranging between 0.0125 µmol/g of the membrane and 1500 µmol/g of the membrane. A concentration of Calcium and Zinc higher than 1500 µmol/g of the membrane can be loaded in the hydrolysed hydrophilic nonwoven nanofibers membrane, when all the accessible COOH groups are coordinated. The excess of Zn^{2+} or Ca^{2+} is physically adsorbed on the membrane's surface in the form of their respective salts (ZnCl_2 and CaCl_2) during the drying of the membrane

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Doxycycline (DOX) was bound non-covalently into membrane by acid-base interactions between amine groups of DOX and carboxyl groups of the membrane as well as by hydrogen bonds between the hydroxyl groups of the membrane. When all the carboxyl and hydroxyl groups available in the membrane are bound to doxycycline, the excess of DOX is physically adsorbed on the membrane's surface during drying. The

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concentration of DOX is ranging between 0.01 mg/mg of the membrane and 1 mg/mg of the membrane.

5 Another aspect of the invention refers to a non-resorbable membrane for promoting bone regeneration characterised in that it comprises the hydrophilic nonwoven nanofibers membrane mentioned above.

10 Another aspect of the invention refers to a non-resorbable periodontal membrane characterised in that it comprises the hydrophilic nonwoven nanofibers membrane mentioned above.

15 Resorbable and non-resorbable barrier membranes are commercially available, being non-resorbable PTFE membranes the Standard of care in Guided Bone Regeneration. The main disadvantage of resorbable membranes is the unpredictable resorption time and toxic substances liberated during degradation, affecting bone formation. Among many others, the main disadvantage of non-resorbable barrier membranes is that they do not osseointegrate. Moreover, in the case of these latest membranes, it is necessary a second surgical intervention to remove them after regeneration that may result in injury of the regenerated tissue. Their poor efficacy results in a high degree of relapse.

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In the present invention the non-resorbable membrane of the invention is a breakthrough bioactive membrane which allows:

- Fully osseointegration, avoiding the need for a second surgery
- 25 • Fast bone regeneration through enhancing the precipitation of natural minerals and the activation of bone-forming cells. No need for filling with bone precursors
- Reducing the proliferation of periodontal bacteria when comprises an antibacterial agent

30 The last aspect of the invention refers to a coating for an implant characterised in that it comprises the hydrophilic nonwoven nanofibers membrane mentioned above which may provide an advantage in osseointegration. Osseointegration involves direct contact between for instance titanium implant and bone. Most metal transcutaneous implants have failed, primarily owing to infection. Titanium alloy implants produce corrosion particles and fail by mechanisms generally related to surface interaction on

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bone to promote an inflammation with fibrous aseptic loosening or infection that can

require implant removal. Further, lowered oxygen concentrations from poor vasculature at a foreign metal surface interface promote a build-up of host-cell-related electrons as free radicals and proton acid that can encourage infection and inflammation to greatly influence implant failure. Covering the implant with the hydrophilic nonwoven nanofibers membrane mentioned above is an efficient way for avoiding the mentioned risks.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skilled in the art to which this invention belongs. Methods and materials similar or equivalent to those described herein can be used in the practice of the present invention. Throughout the description and claims the word "comprise" and its variations are not intended to exclude other technical features, additives, components, or steps. Additional objects, advantages and features of the invention will become apparent to those skilled in the art upon examination of the description or may be learned by practice of the invention. The following examples and drawings are provided by way of illustration and are not intended to be limiting of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Theoretical modelling of co-polymerization of MA and HEA, F_a vs conversion (A) and F_a vs f_a (B).

FIG. 2. Chromatographic profile of HEA/MA-10/90 (A), HEA/MA-15/85 (B), HEA/MA-25/75 (C), HEA/MA-35/65 (D), HEA/MA-45/55 (E).

FIG. 3. ^1H RMN spectra of HEA/MA-10/90 (A), HEA/MA-15/85 (B), HEA/MA-25/75 (C), HEA/MA-35/75 (D)

FIG. 4. Theoretical modelling of co-polymerization of MMA and HEMA, F_a vs conversion (A) and F_a vs f_a (B).

FIG. 5. Chromatographic profile of MMA-co-HEMA (A), and ^1H RMN spectra of MMA-co-HEMA (B).

FIG. 6. Electrospinning set up: injection pump (1), injection needle (2), Drum collector (3), high voltage sources (4), Taylor cone display (5) and mechanical axis with transversal movement (6).

5 **FIG. 7.** Nonwoven mat produced with the blends: (A)/(B) 0:100 (A), 100:0 (B), 25:75 (C), 50:50 (D), 75:25 (E).

FIG. 8. Nonwoven mats produced with the blend (A)/(B)75:25 (A), and with the blend (A)/(B)50:50 (B)

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FIG.9. Tiss-OH before (A) and after heating (B).

FIG.10. Connective collagen network (A), and nonwoven nanofibers mat (Tiss-OH) (B).

15 **FIG. 11.** FESEM micrographs of membranes after 7 days of immersion in SBFS; TissHYD (A), Tiss-Ca²⁺ (B), Tiss-Zn²⁺ (C).

FIG. 12. Bone histomorphometry obtained after using Tiss-Zn²⁺, by coloration with von Kossa silver nitrate to visualize mineralized bone, at six weeks of follow up: histology section including the bone defect and the region of interest (ROI) showing a large formation of dense bone (A). Total surface (TS) at ROI; asterisks (*) show the presence of marrow and adipose-like tissue (B); bony bridging (BB) images are observed. Bone perimeter (BPm) at ROI (C). Bone thickness (BTh) with the traced measurements at ROI (D). Osteoid surface (OS), ROI (E).

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FIG. 13. Bone histomorphometry obtained by coloration with von Kossa silver nitrate to visualize mineralized bone, at six weeks of follow up, after using no membrane- control (A) and Tiss-Ca²⁺ (B). Trabecular bone formation were observed along the margin of calvarial defect (arrow head), and within the defect. Mbr: membrane, NB: new bone and OB: old bone (pointers show scattered bone islands, in correspondence with new bone).

30 **FIG. 14.** Bone histology obtained after using Tiss-Zn²⁺ (A) and Tiss-Ca²⁺ (B) membranes by coloration with toluidine blue to visualize mineralized bone, at six weeks of healing time. Single arrows indicate the presence of osteoblasts; double arrows

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indicate the presence of osteocytes; faced arrows mean blood vessels; pointers indicate fibrous connective tissue. NB: new bone, Os: osteoid tissue.

FIG.15. Field Emission scanning electron microscopy (FE-SEM) of *F. nucleatum*, *S. oralis*, *A. naeslundii*, *V. parvula*, *A. actinomycetemcomitans* and *P. gingivalis* grown as multi-species biofilm in vitro at 12 hours of incubation on, PTFE (control) (A), hydroxyapatite (HAp) discs (B) TissHYD (C), Tiss-Ca²⁺ (D), Tiss-Zn²⁺ (E) and Tiss-DOX (F).

FIG.16. Number of bacteria [Log CFU/biofilm mean (standard deviation)] of *F. nucleatum*, *S. oralis*, *A. naeslundii*, *V. parvula*, *A. actinomycetemcomitans* and *P. gingivalis* grown as multi-species biofilm in vitro at 72 hours of incubation, measured by quantitative real-time polymerase chain reaction (qPCR) (N=3 for each incubation time) in Hydroxyapatite discs (HAp), PTFE (control), T-COOH, Tiss-Ca²⁺, Tiss-Zn²⁺ and Tiss-DOX.

Fig.17. FESEM micrographs of tissues after silicon dioxide doping and SBFS immersion for 7 days: Tiss-SiO₂-COOH (A), Tiss-SiO₂-Ca²⁺ (B), Tiss- SiO₂-Zn²⁺ (C) and Tiss- SiO₂-DOX (D).

EXAMPLES

1. COPOLYMERS SYNTHESIS

1.1 Preparation of (MA-co-HEA) copolymers

In the present invention a variant of Metal Catalysed Living Radical Polymerization (MC-LRP): Copper-mediated Living Radical Polymerization (Cu⁰-MC-LRP) has been optimised to obtain hydrophilic acrylate lineal (MA-co-HEA) copolymer with statistical topology and high molecular weight (above 1x10⁶ Da).

The Cu⁰-LRP system used was: Methyl 2-bromopropionate as initiator, Tris(2-dimethylaminoethyl)amine as ligand, Copper/Copper(II) as transition metal: MBP/M₆-TREN/Cu⁰/BrCu₂, and dimethyl sulfoxide (DMSO) was used as solvent. The monomers selected were: methyl acrylate (MA), and hydroxyethyl acrylate (HEA).

First a theoretical analysis of the co-polymerization of MA and HEA was done by using the terminal model [Mayo, F. R.; Lewis, F. M. *J. Am. Chem Soc.* 66, (1944), 1594–

1601]. The terminal model assumes that radical reactivity only depends on the terminal unit of the growing chain, such that the molar fraction of monomer-a in the copolymer (F_a) depends only of monomer mole fractions (f_a and f_b , with $f_a + f_b = 1$) and the monomer reactivity ratios:

$$F_a = \frac{r_a f_a^2 + f_a f_b}{r_a f_a^2 + 2 f_a f_b + r_b f_b^2}$$

where r_a and r_b are the co-polymerization Reactivity Ratios $r_a = kp_{aa}/kp_{ab}$, $r_b = kp_{bb}/kp_{ba}$; kp_{ij} is the propagation rate coefficient for addition of monomer- j to radical- i . The co-polymerization Reactivity Ratios for MA and HEA used are $r_a=0.94$ and $r_b=0.90$ respectively.

Fig. 1 shows the theoretical modelling of co-polymerization of MA and HEA: F_a vs conversion (F_a is the molar fraction of MA in the copolymer along the Polymerization) for different initial molar fractions feed of MA (f_{0a}), and F_a vs f_a (f_a is the molar fraction of MA in the feed along the Polymerization).

Theoretical modelling of co-polymerization of MA and HEA shows that F_a is practically equal to the initial molar fraction of feed (**Fig. 1A**) for any initial molar fractions of MA (**Fig. 1B**). The system runs through an almost azeotropic co-polymerization to any initial molar fraction of the feed. Therefore, theoretically the co-polymerization of MA and HAE provide copolymer with statistical topology (MA-HEA-MA-HEA-MA-HEA.....).

Cu⁰-LRP is very sensitive to any trace of impurities: mainly the inhibitor that contains both monomers, and di-acrylates that are formed in monomer HEA by condensation of HEA molecules. Thus, in Cu⁰-LRP the presence of impurities at very low concentration provides low yields, low molecular weights and crosslinked polymer, and thus it is strictly necessary to properly purify the monomers.

HEA purification protocol:

1. First HEA was purified in a basic alumina column.
2. 70 ml of HEA previously purified on basic alumina column, were dissolving in 210 ml of distilled water, and then the traces of ethylene glycol diacrylate were eliminate by 11 liquid-liquid extractions with 210 ml of hexane.

3. Then 58 g of NaCl were dissolved in the HEA aqueous solution, and HEA monomer was extracted by 5 liquid-liquid extractions with 200 ml of diethyl ether.
4. The diethyl ether HEA solution was dried with 300g of anhydrous sodium sulphate: the solution was stirred a few minutes and then was filter to remove the sodium sulphate. .
5. Then the diethyl ether was completely evaporated in a rotavapor, and the purified HEA was stored at -20°C.

MA purification protocol:

1. The required volume of MA was passed through a column of basic alumina.

Once the monomers were purified, six (MA-co-HEA)_copolymers with different molar % of HEA and MA in the feed were synthesised by Cu⁰-LRP . The six different HEA/MA molar % were: a) HEA/MA 10/90 b) HEA/MA 15/85, c) HEA/MA 25/75, d) HEA/MA 34/66, e) HEA/MA 45/55, f) HEA/MA 55/45. **Table 1** shows the % wt of each component in the final polymerization mixture for each molar % of HEA and MA.

Table 1. % wt of each component in the mixture for each molar % of HEA and MA.

% molar HEA/MA	HEA % wt	MA % wt	DMSO % wt	MBP % wt	Cu ⁰ % wt	M6- TREN % wt	CuBr ₂ % wt
a) 10/90	6.2040	43.7843	49.9900	0.0050	0.0017	0.0140	0.0010
b) 15/85	9.7020	40.2863	49.9900	0.0050	0.0017	0.0140	0.0010
c) 25/75	15.5630	34.4253	49.9900	0.0050	0.0017	0.0140	0.0010
d) 34/66	20.3690	29.6193	49.9900	0.0050	0.0017	0.0140	0.0010
e) 45/55	23.8780	26.1103	49.9900	0.0050	0.0017	0.0140	0.0010
f) 55/45	28.8170	21.1713	49.9900	0.0050	0.0017	0.0140	0.0010

The total mass of the monomers (MA + HEA) 59.2700 g was added into 50 ml Schlenk flasks, and then were added: 59.2700 g of DMSO, 0.0020 g of Cu⁰, 0.0160 g of Tris[2-(dimethylamino)ethyl]amine (M₆-TREN), 0.0012 g of CuBr₂, and 0.0060 g of methyl 2-bromopropionate (MBP). The flasks were closed with a septum, the oxygen was removed by bubbling nitrogen for few minutes, and then four freeze-pump-thaw cycles were done (after the last freeze-pump-thaw cycle the flasks were filled with nitrogen).

Then the sealed flask was placed in a thermostatic oil bath at 25 °C during 24h. Then the copolymers were purified by dissolving in acetone and precipitating them in distilled water (two times). After purification copolymers were dried in a vacuum at 80° C to a constant weight. The copolymers a), b), c), d) had a white colour and rubber texture, and the final conversion was between 90-95%wt in all the cases. The copolymerization of e) and f) (**Table 1**) did not occur properly: the yield was below 40%, and the copolymer had not a rubber texture. Therefore the optimal range of molar % of HEA in the feed was between 10% and 34%.

(MA-co-HEA) copolymers were characterised by GPC (Viscotek 270max of Malvern) and by ¹H RMN (Bruker Avance 400 MHz spectrometer). The samples for GPC were prepared dissolving 1 mg of copolymers in 10 ml 1-methyl-2-pyrrolidinone (NMP) and they were analysed in triplicate.

Fig. 2 shows a chromatographic profile of each copolymers, and **Table 2** shown the molecular weight (M_w and M_n) and M_w/M_n . **Fig. 3** shows the ¹H RMN spectra of said copolymers.

Table 2. Molecular weights of each synthesised copolymer.

MA-co-HEA-10/90 M_w (Da)	MA-co-HEA-10/90 M_w (Da)	MA-co-HEA-10/90 Average M_w (Da)	MA-co-HEA-10/90 Average M_n (Da)	M_w/M_n
1.162x10 ⁶	716449	1.156x10 ⁶ ± 40847	777591 ± 53003	1.49
1.137x10 ⁶	805809			
1.168x10 ⁶	810516			
MA-co-HEA-15/85 M_w (Da)	MA-co-HEA-15/85 M_w (Da)	MA-co-HEA-15/85 Average M_w (Da)	MA-co-HEA-15/85 Average M_n (Da)	M_w/M_n
6.039x10 ⁶	2.155x10 ⁶	5.746x10 ⁶ ± 653911	1.878x10 ⁶ ± 395400	3.08
5.671x10 ⁶	1.721x10 ⁶			
5.529x10 ⁶	1.759x10 ⁶			
MA-co-HEA-25/75 M_w (Da)	MA-co-HEA-25/75 M_w (Da)	MA-co-HEA-25/75 Average M_w (Da)	MA-co-HEA-25/75 Average M_n (Da)	M_w/M_n
3.167x10 ⁶	1.815x10 ⁶	3.188x10 ⁶ ± 88.220	1.714x10 ⁶ ± 220000	1.86
3.168x10 ⁶	1.714x10 ⁶			
3.229x10 ⁶	1.614x10 ⁶			

<i>MA-co-HEA-34/66 M_w (Da)</i>	<i>MA-co-HEA-35/65 M_w (Da)</i>	<i>MA-co-HEA-35/65 Average M_w (Da)</i>	<i>MA-co-HEA-35/65 Average M_n (Da)</i>	<i>M_w/M_n</i>
1.980x10 ⁶ 1.981x10 ⁶ 1.957x10 ⁶	1.413x10 ⁶ 1.418x10 ⁶ 1.355x10 ⁶	1.972x10 ⁶ ± 33729	1.395x10 ⁶ ± 35019	1.41

Table 3 shows the real molar % of HEA in each copolymer: it was calculated by the intensity ratio between the signals **a** (CH₃ of MA) and **b** (CH₂–CH₂ of HEA) of ¹H RMN spectra.

Table 3. Real molar % of HEA in each synthesised copolymer.

Ref	Integral signal b	Integral signal a	(MA) _x -co-(HEA) _y	Molar % HEA in the copolymer
MA-co-HEA-10	0.44	3.01	(MA) ₉ -co-(HEA) ₁	10
MA-co-HEA-15	2.43	10.76	(MA) ₆ -co-(HEA) ₁	14
MA-co-HEA-25	2.24	4.39	(MA) ₅ -co-(HEA) ₂	28
MA-co-HEA-35	2.15	2.81	(MA) ₃ -co-(HEA) ₂	36

- 5 The analysis of ¹H RMN shows that the concentrations of HEA in the copolymers are practically the same as the feed concentrations.

The solubility of synthesised acrylate copolymers was tested in acetone, dimethyl formamide (DMF), dimethyl sulfoxide (DMSO), 1-4 dioxane and NMP. The copolymers were totally soluble in all the tested solvents up to 6% wt: above 6% the viscosity of the solutions was extremely high. The 6% wt solution with lower viscosity was the DMF solution, which indicates that DMF is the best solvent for these copolymers.

In order to have the maximum concentration of functional groups (OH groups) in the nonwoven nanofibers mat, the selected acrylate copolymer for the blend formulations was (MA)₃-co-(HEA)₂ (**Table 2**).

15 **1.2 Preparation of MMA-co-HEMA copolymers**

The theoretical modelling of co-polymerization of MMA and HEMA with statistical topology (MMA-HEMA-MMA-HEMA-MMA-HEMA.....) is shown at **Fig. 4**: F_a vs conversion wherein F_a is the molar fraction of MMA in the copolymer along the polymerization for different initial molar fractions feed of MMA (f_{0a}), and F_a vs f_a wherein f_a is the molar fraction of MMA in the feed along the Polymerization.

The Cu⁰-LRP technique used to synthesise the MA-co-HEA copolymers did not work well in the co-polymerization of the methacrylic monomers (MMA and HEMA): very low yields, and molecular weights were obtained, and also the concentration of HEMA in the copolymers was much lower than feed concentrations.

- 5 Therefore, in order to synthesise a methacrylate copolymer chemically miscible with (MA)₃-co-(HEA)₂, the co-polymerization of MMA and HEMA was carried out by another variant of MC-LRP how is reverse Atom Transfer Radical Polymerization (reverse-ATRP) [*Chem. Rev.* 101,(2001),2921-2990.]: Molecular weight as high as in Cu⁰-LRP cannot be obtained by reverse ATRP, but reverse-ATRP is much less sensitive to
10 impurities, and thus much easier to carry out.

The reverse-ATRP system used was: 2,2'-azobis(2-methylpropionitrile) (AIBN) as initiator, *N,N,N',N',N'*-pentamethyldiethylenetriamine (PMDETA) as ligand, copper(II) as transition metal, and a mixture of dimethyl sulfoxide (DMSO)/xylene was used as solvent. The selected monomers were methyl methacrylate (MMA), and 2-
15 hydroxyethylmethacrylate (HEMA).

Protocol of purification of MMA and HEMA:

1. The required volume of MMA and HEMA was passed through a column of basic alumina.

In a 500ml two-necked flask equipped with a reflux and magnetic stirrer were added:
20 95.00ml of DMSO, 0.14g CuBr₂ 0.24g of *N,N,N',N',N'*-pentamethyldiethylenetriamine (PMDETA), 90.08g of MMA and 40.12g of HEMA previously purified, 0.23g of 2,2'-azobis(2-methylpropionitrile) (AIBN) dissolved in 60.02 g of xylene. The mixture was stirred at 250 rpms, when all the components were completely dissolved the reaction mixture was cooled at 0° C and purged with highly pure nitrogen for 20 min. Then the
25 reaction was carried out at 80 °C in an oil bath for 6 hour. After polymerization, the copolymer was purified by dissolving in acetone and precipitating it in distilled water three times. Then the solid copolymer was washing with distilled water 3 times, and dried in a vacuum at 80° C to a constant weight. The methacrylate copolymer had a white colour and a hard and brittle texture. The conversion was 70%.

30 *Weight % of each component in the co-polymerization mixture:* 33.200% DMSO, 0.050% CuBr₂, 0.083% PMDETA, 14.030% HEMA, 31.500% MMA, 0.080% AIBN and 21.000% xylene.

The MMA-co-HEMA copolymer was characterised by GPC (Viscotek 270 max of Malvern) and by ^1H RMN (Bruker Avance 400 MHz spectrometer). The samples for GPC were prepared dissolving 1 mg of copolymers in 10 ml 1-Methyl-2-pyrrolidinone (NMP) and they were analysed in triplicate. **Fig. 5** shows the chromatographic profile and ^1H RMN spectrum of the prepared MMA-co-HEMA copolymer.

Table 4 shows molecular weights M_w and M_n and M_w/M_n calculated by GPC.

Table 4. Molecular weights of MMA-co-HEMA copolymers.

M_w (Da)	M_n (Da)	Average M_w	Average M_n	M_w/M_n
229000	134000	230855 ± 7418	115748 ± 35567	2.02
229266	113267			
234300	99977			

Furthermore, **Table 5** shows the real molar % of HEMA in the copolymer calculated by the intensity ratio between the signals **a** (CH_3 of MMA) and **b** ($\text{CH}_2\text{--CH}_2$ of HEMA) of the ^1H -NMR spectra.

Table 5. Real Molar % of HEMA in the copolymers.

Ref	Integral signal b	Integral signal a	$(\text{MMA})_x\text{-co-}(\text{HEMA})_y$	Molar % HEMA in the copolymer
MMA-co-HEMA	1.73	1.72	$(\text{MMA})_1\text{-co-}(\text{HEMA})_1$	50

The solubility of $(\text{MMA})_1\text{-co-}(\text{HEMA})_1$ was tested in acetone, dimethyl formamide (DMF), dimethyl sulfoxide (DMSO), 1-4 dioxane and NMP, and the copolymer was totally soluble up to a percentage of 38% wt; above 38% the viscosity of the solutions was extremely high. For $(\text{MMA})_1\text{-co-}(\text{HEMA})_1$ the 38% wt solution with lower viscosity also was DMF solution, which indicates that DMF is also the best solvent for this copolymer.

2. PREPARATION OF NONWOVEN MAT BY ELECTROSPINNING

The solubility between copolymers $(\text{MMA})_1\text{-co-}(\text{HEMA})_1$; (A) and $(\text{MA})_3\text{-co-}(\text{HEA})_2$; (B) was tested in acetone, dimethylformamide (DMF), dimethyl sulfoxide (DMSO), 1-4 Dioxane and NMP. The (A)/(B) w/w ratios tested were: 10/90, 25/75, 50/50, 75/25 and 90/10, and the ratio $((A)+(B))/\text{solvent}$, w/w was 3/97 in all the cases. Both copolymers were completely soluble in all the solvents, at all of ratios. The solutions of lower

viscosity were DMF solutions, and thus DMF was selected as solvent to optimize the electrospinning process.

In order to study and modulate the mechanical properties of nonwoven mats, the selected blends; (A)/(B) w/w to be process by electrospinning were: 0/100, 25:75, 50:50, 75:25 and 100/0, and blend/solvent, w/w was 3/97. When the copolymers were completely dissolved the solutions were loaded into 20 cm³ teflon syringes (Becton & Dickinson) and extruded through a stainless-steel capillary tube with outer and inner diameters of 1.5 mm and 1.1 mm, respectively. The injection system was coupled to a mechanical system with axial movement, the flow rates and voltages were selected in order to allow the collection of dry fibers in nonwoven mats, and the fibers were collected on a rotary drum collector. **Fig. 6** shows the electrospinning set up, and **Table 6** shows the electrospinning processing parameters.

Table 6. Electrospinning processing parameters.

Parameter	Value
Potential difference (ΔV)	10 kV
Flow rate (Q)	0.5 ml/h
Drum rotation speed (ω)	500 rpm
Distance between electrodes (δ)	26 cm
Temperature (T)	20-25°C
Relative humidity (H.R.)	25-30%
Processing time (t)	2 h
Axis movement (mm)	± 150

Fig. 7 shows a SEM analysis of nonwoven nanofibers mats obtained with the blends (A)/(B) 0:100 (A), 100:0 (B), 25:75 (C), 50:50 (D), 75:25 (E).

The copolymer (B) pure provided a very elastic gummy materials, in which the fibers are 100% fused together forming a film (**Fig. 7A**).

The nonwoven mats obtained whit the copolymer (A) pure, did not have any fusion point between fibers, they were completely loose (**Fig. 7B**), and this provided materials with a null abrasion resistance; hardly manipulable materials.

In the nonwoven mats obtained with the blend (A)/(B) 25:75 (**Fig. 7C**), the fusion between fibers was no longer 100% as in the case the copolymer (B) pure (**Fig. 7A**), but there were still many fusion points between fibers (black circles in **Fig. 7C**), and

thus nonwoven mats with very low specific surface, and very high elasticity were obtained.

The blend (A)/(B) 75:25 (**Fig. 7E**) provided a compact nonwoven mats with better abrasion resistance than the obtained with the copolymer (A) pure (**Fig. 7B**), but the mats were not yet flexible, and they showed a very low resistance to stress.

The blend (A)/(B) 50:50 w/w (from now called **Tiss-OH**; **Fig. 7D**) provided a compact nonwoven mats with excellent mechanical properties: very high abrasion resistance, high flexibility, high elasticity, high stress resistance, and thus it is easily to manipulated: can be cut, bend, twist ... etc.

The low abrasion resistance of nonwoven mat produced with the blend (A)/(B)75:25, compared with the nonwoven mat produce with blend (A)/(B)50:50 is shown in the **Fig. 8**.

Electrospinning scaling process of Tiss-OH

In order to increase **Tiss-OH** production, the ratio blend/solvent (DMF) w/w was studied. The optimum blend/DMF w/w % was 13.50/86.50: above it the viscosity was too high to be process by electrospinning.

In order to keep the electrospinning process stable over the time, it was necessary to increase the conductivity of the solution blend/solvent by addiction of 0.048g of hydrochloric acid (HCl).

The optimal solution blend/solvent for scaling the electrospinning process was prepared as follows: 5.000g of (MA)₃-co-(HEA)₂ (6.246% wt) and 5.000 g of (MMA)₁-co-(HEMA)₁ (6.246% wt) were dissolve in 70.000g of DMF (87.4479% wt), when the copolymers were completely dissolve, 0.048g of hydrochloric acid (HCl) (0.059% wt) were added to the solution.

The electrospinning set up was the same as the one shown in **Fig. 6**, but in order to increase the production the injection system of a single needle of **Fig. 6** was replaced by a ten-needle head.

The optimized electrospinning parameters for a ten-needle head are shown in the **Table 7**.

Table 7. Scaling electrospinning parameter

Parameter	Value
Potential difference (ΔV)	8 kV
Flow rate (Q)	5.0 ml/h
Drum rotation speed (ω)	500 rpm
Distance between electrodes (δ)	15 cm
Temperature (T)	20-25°C
Relative humidity (H.R.)	25-30%
Processing time (t)	2-10 h
Axis movement (mm)	± 150

The thickness of Tiss-OH was easily controlled from a few microns to hundreds of microns, by controlling the processing time (2h of processing $\approx 45 \mu\text{m}$ in thickness).

2.1. Conversion of Tiss-OH from hydrophobic to hydrophilic

Initially Tiss-OH is hydrophobic; to convert it into hydrophilic material is necessary to carry out a further thermal treatment. The thermal treatment was done by introducing Tiss-OH, in hot water (40°C) for 5 hours. To prevent the shrinking of the materials during the thermal treatment, they were kept tensioned using frames.

The thermal treatment produces an irreversible reorientation of the hydrophobic and hydrophilic domains present on the fibers surface, causing the material to go from being completely hydrophobic to being highly hydrophilic: the OH groups of the fibers are reordered in order to interact by hydrogen bonding with the water molecules, while the hydrophobic groups hide from the water.

After thermal treatment the water adsorption capacity (Q) of Tiss-OH, was calculated taking into account the expression:

$$Q = (\text{Absorbed mass of water}) / (\text{Mass of dry nonwoven mat})$$

Six nonwoven mat samples of different mass were dried in a vacuum oven at 50°C during 2h. Then, they were submerged in distilled water for 3h at room temperature; the water retained in the surface of the samples was removed using a cellulose paper. Subsequently, the samples were weighed and the Q calculated was 2.06 ± 0.15 .

Moreover, in order to estimate the pore size distribution of Tiss-OH roughly, a vacuum filtration test using a series of aqueous suspensions of monodisperse hydrophilic nano

and microparticles with sizes between 150nm and 5000nm of diameter has been performed. Tiss-OH allowed passing particles from 800nm to 3000nm of diameter.

The thermal resistance of Tiss-OH, was studied by immersing it in water at 100°C during 24h. The internal structure, mechanical properties and the mass of Tiss-OH were exactly the same before and after heating (**Fig. 9**).

2.2. Hydrolisis of Tiss-OH to get Tiss-HYD

Natural collagen mesh that forms the connective tissue of bones is composed of nanofibrils of approximately 50nm that are grouped to form fibers of approximately 500nm with a similar morphology, mechanical and physical-chemical properties to those of Tiss-OH.

Fig. 10 SEM pictures of connective collagen network (A) and Tiss-OH (B), that showing the very similar morphological structure.

In order to introduce carboxyl groups (COOH) on the fibers surface, a partial hydrolysis of ester groups: $R-COOCH_3$ and $RCOOCH_2CH_2OH$ of the membrane Tiss-OH of 45 microns of thickness (**Fig. 7D**), was carried out to obtain TissHYD. The hydrolysis solution was sodium carbonate (333 mM), pH=12.50. It was found that a hydrolysis time longer than 1 hour, produced a high rigidity in the membranes, making them fragile and brittle. Therefore the time selected for the hydrolysis was 30 min. Therefore the hydrolysis was done by introducing Tiss-OH in a solution of sodium carbonate (333 mM), pH=12.50 during 30 min. Then, the membranes (TissHYD) were washed 3 times with distilled water and dry at room temperature. The number of COOH groups calculated by the toluidine blue O adsorption assay (TBO method) according to *Biomaterials*.14, (1993), 817-822. The assay includes the incubation of carboxylated matrixes with toluidine blue O in alkaline buffer with subsequent washing, followed by elution and quantification of eluted TBO via UV-Vis spectrometry. The number of accessible carboxyl groups was 560 ± 50 $\mu\text{mol/g}$ of the membrane. After the hydrolysis the Q calculated was 3.06 ± 0.20 .

2.2. Functionalization of TissHYD with Zn^{+2} (Tiss- Zn^{2+}) and Ca^{2+} (Tiss- Ca^{2+})

TissHYD was functionalised with Zn^{+2} (Tiss- Zn^{2+}) and Ca^{2+} (Tiss- Ca^{2+}). The ability of carboxyl groups to complex divalent cations was used to functionalise TissHYD (Tiss-membranes) with Zn^{+2} and Ca^{2+} . TissHYD was soaked with a Zn^{2+} and Ca^{2+} solution,

and then the water was evaporated in a vacuum at constant temperature: by this way TissHYD was loaded with 1.1 $\mu\text{g}/\text{mg}$, of Ca^{2+} and Zn^{2+} .

2.3. Functionalization of TissHYD with doxycycline (Tiss-DOX)

5 Doxycycline (DOX) was bound non-covalently into TissHYD by acid-base interactions between amino groups of DOX and carboxyl groups of TissHYD. TissHYD was soaked with twice its mass of a DOX solution of 40 mg/ml, and then the water was evaporated in a vacuum at constant temperature: by this way TissHYD was loaded with 0.8 mg of DOX/mg Tiss.

3. ACELLULAR STATIC IN VITRO BIOACTIVITY TEST OF TissHYD, Tiss- Zn^{2+} , Tiss- Ca^{2+}

Membranes should enhance bone formation through bioactivity, therefore for said application analysis proposed by Kokubo has been performed (ISO 23317:2012. Implants for surgery. In vitro evaluation for apatite-forming ability of implant materials).

15 The membranes were soaked in 20 ml of simulated body fluid solution (SBFS) [pH 7.45] in sterile flasks for 7 days. Reagents per 1000 ml of SBFS were: 8.035 g of NaCl, 0.355 g of NaHCO_3 , 0.225 g of KCl, 0.231 g of $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 0.311 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 39 g of 1M HCl, 0.292 g of CaCl_2 , 0.072 g of Na_2SO_4 , 118 g of Tris, 0 to 5 ml of 1M HCl for final pH adjustment.

20 After drying, surfaces were analyzed by FESEM at 2.5 Kv, 3.5 mm working distance and elemental analysis was done by means of an EDX attached to the FESEM, at a working distance of 15 mm. Results from FESEM images of TissHYD, Tiss- Ca^{2+} and Tiss- Zn^{2+} after 7 days of immersion in SBFS are presented in **Fig. 11**.

After immersion, differences between groups were evidenced:

25 In TissHYD (**Fig. 11A**) some rounded deposits were observed rarely on the samples. Traces of calcium were found at the EDX spectra.

In Tiss- Ca^{2+} (**Fig. 11B**), an increase in nanofiber diameter was found, and nanofiber lost their smooth appearance. Spotty calcium deposits were uniformly distributed throughout nanofibers surfaces.

In Tiss-Zn²⁺ (**Fig. 11C**), nanofiber diameter was highly increased (from 300 to about 500nm), and deposits of mineral (100nm) were also randomly distributed onto the nanofibers surfaces.

Calcium and phosphorous were encountered at the EDX spectra on nanofibers surfaces. Numerous agglomerations of spherical nanocrystals were identified onto the Tiss-Zn²⁺ surface. SBFS are fluids with ion concentrations nearly equal to those of human blood plasma and are employed for evaluating the bioactivity of biomaterials for hard tissue repair. Zinc promoted biomimetic precipitation of Ca/P deposits and formation of Hydroxyapatite (HAp, Ca₁₀(PO₄)₆(OH)₂) nanocrystals during SBFS immersion. Zinc complexation on tissues facilitated phosphate groups binding. These phosphate groups, at the surface, have under-coordinated oxygens, which lead to reactive surfaces that will attract calcium ions from SBFS. This biomimetic deposition of Ca/P is considered as a coating method inspired by the natural process of biomineralization. Moreover, it should be considered that crystalline HAp is very slow to resorb, and most bone substitutes based on HAp do not resorb or resorb extremely slowly. However, if HAp or nano-HAp is precipitated onto the surfaces, it does resorb, facilitating hard tissue regeneration. Biomimetic remineralization of the tested tissues will facilitate bone regeneration. HAp facilitates formation of other bone apatite-like materials as carbonate HAp and it is able to stimulate cells, leading to the formation of bone. Moreover, HAp promotes osteoconductivity. Osteoblasts stimulated with extracellular Ca²⁺ and PO₄²⁻ increased bone morphogenetic protein-2 mRNA expression. Fibroblast growth factor-2 (FGF-2) gene and protein expression levels are also augmented by increases in extracellular Ca²⁺ concentration.

4. BONE FORMATION IN A RABBIT CALVARIAL DEFECT MODEL

Three types of membranes were tested Tiss-Zn²⁺ (loaded with 1.1 µg(Zn²⁺)/mg Tiss), Tiss-Ca²⁺ (loaded with 1.1 µg(Ca²⁺)/mg Tiss) and TissHYD. Naked defects without any type of membrane were used as a control. Six white, New Zealand-breed experimentation rabbits with identical characteristics (age: 6 months; weight: 3.5–4 kg) were selected for the study and fed daily with rabbit-maintenance Harlan-Teckland Lab Animal Diets (2030). The surgical interventions were carried out at the Minimally Invasive Surgery Centre Jesús Usón (CCMI, Cáceres, Spain). The experiment was developed in accordance with the guidelines of the US National Institute of Health (NIH) and European Directive 86/609/EEC regarding the care and use of animals for experimentation. The study also complied with the European Directive 2010/63/EU about the protection of animals used for scientific purposes and with all local laws and

regulations. The researchers obtained the approval of the Ethics Committee of the Institution. As required by the legislative framework, the minimum number of animals was used for ethical reasons. Comparable models have been published concerning the histological and animal experimentation methods.

5 Before starting the surgical procedure, vital signs were taken and then immobilisation of the rabbits was carried out. Midazolam (0.25 mg/kg) and propofol (5 mg/kg) were administered intravenously as anaesthetics for induction and an inhalation of 2.8% inspired sevoflurane gas was also used. Analgesia was provided with ketorolac (1.5 mg/kg) and tramadol (3 mg/kg). Once the animals were sedated and ready, with a No.

10 15 scalpel blade incisions were made between the bases of their ears and between their eyes. A surgical triangular field was done after connecting the two incisions with another one in the skull midline. With a Prichard periosteotome, the epithelial, connective, and muscular tissues were separated from the operation field and the skull surface was washed with a sterile saline solution. Six non-critical bone defects

15 (diameter: 6 mm; depth: 3 mm) were created on the parietal bone, on each side of the skull midline, 3 mm apart, using a trephine (Helmut-Zepf Medical GmbH, Seitingen, Germany) mounted on an implant micromotor operating at 2000 rpm under saline irrigation. The trephine had an external diameter of 6 mm, a length of 30 mm, and teeth of 2.35 mm. Piezosurgery was used to remove the inner table and the medullary bone

20 in every defect. The depth was controlled with a periodontal probe. A randomly assigned membrane was used to cover each bone defect, leaving a naked defect in each animal. The randomisation sequence was generated using specific software (Research Randomizer, V. 4.0, Urbaniak GC & Plous S, 2013). The membranes were fixed with the fibrin tissue adhesive Tissucol (Baxter, Hyland S.A. Immuno, Rochester,

25 MI, USA), which was placed on the bone rims adjacent to the defects. Proper adhesion and limited mobility of the membranes were confirmed when the flaps were moved back to their initial positions. Sutures were made on the following planes using resorbable material: periosteal (4/0), sub-epidermal (4/0) and skin (2/0). Simple stitches were used as close as possible to the edge. The wound was carefully cleaned with a

30 sterile saline solution. Anti-inflammatory analgesia (buprenorphine 0.05 mg/kg and carprofen 1 mL/12.5 kg) was administered. The animals were sacrificed six weeks after surgery using an intravenous overdose of potassium chloride solution. Samples were obtained from the skull of each specimen, cutting them in an anatomical sagittal plane. After the brain mass was separated and the skull was washed with a sterile saline

35 solution, the tissue samples were cut and marked individually.

Specimens in cranial blocks were recovered and stored in a 5% formaldehyde solution (pH 7) and blocks were retrieved from the regenerated bone defect using an oscillating autopsy saw (Exakt, Kulzer, Wehrheim, Germany). The dissected specimens were immediately immersed in a solution of 4% formaldehyde and 1% calcium and processed for ground sectioning following the Donath and Bruener method. For histological staining and rapid contrast tissue analysis (Merck Toluidine Blue-Merck, Darmstadt, Germany), a metachromatic dye was used to assess the percentage of new bone formation. The von Kossa (VK) silver nitrate technique (Sigma–Aldrich Chemical Co., Poole, UK), by using the software Image J, was applied to visualise the mineralised bone. The following data were compiled: bone surface (BS), osteoid surface (OS), percentage of osteoid surface (OS/TS), bone perimeter (BPm) and bone thickness (BTh). A 1% toluidine blue (TB) solution with a pH of 3.6 was chosen and adjusted with HCl 1 N. The samples were exposed to the dye for 10 minutes at RT, rinsed with distilled water, and air-dried. Osteocytes, osteoblasts and blood vessels were analysed in TB stained sections.

Means and standard deviations (SD) were calculated in pixels, and then converted into mm or mm². One-way ANOVA and paired samples t-tests were applied with a level of significance at $p \leq 0.05$.

The implanted membranes were well tolerated by the surrounding soft tissues, with no evidence of necrosis, allergy symptoms, immune reactions, or incompatibility. All specimens showed no signs of inflammation or infection induced by the use of biomaterials.

The von Kossa (VK) staining technique permitted to observe that all bone defects treated with membranes showed higher bone surface (BS) and bond thickness (BTh) than the control group (**Tables 9 and 10**). In the **Fig. 12** shows bone a bone defect with an implanted Tiss-Zn²⁺ membrane stained with von Kossa silver nitrate to visualize mineralized bone, at six weeks of follow up.

The **Fig. 12A** is a histology section including the bone defect and the region of interest (ROI), showing a large formation of dense bone.

The **Fig. 12B** is the total surface (TS) at ROI; asterisks (*) show the presence of marrow and adipose-like tissue. Bony bridging (BB) images are observed.

At **Fig. 12C**, bone perimeter (BPm) at ROI is observed, and in **Fig. 12D**, bone thickness (BTh) with the traced measurements at ROI is measured.

In the **Fig. 12E**, an osteoid surface (OS), is represented. It can be appreciated as the membranes have higher BTh than control (Ctr), and thus they produced more osteoid surface (OS), in comparison with the control group (see the ratio OS/TS in **Tables 9 and 10**).

Table 9. Histomorphometric data obtained within the new bone formed in the region of interest (ROI) (Mean±SD)

Ref	BS (mm ²)	OS (mm ²)	OS/TS (%)	BPm (mm)	BTh (mm)
Ctr	1.63±0.78	0.31±0.30	0.05±0.05	46.09±19.86	0.58±0.22
Tiss-Zn²⁺	3.27±1.15	0.48±0.24	0.06±0.02	100.33±18.60	1.64±0.23
Tiss-Ca²⁺	2.70±0.82	0.46±0.21	0.05±0.02	82.56±37.69	1.58±0.47
TissHYD	2.52±0.67	0.46±0.20	0.06±0.03	70.37±7.39	1.14±0.37

Abbreviations: BS: Bone Surface, OS: Osteoid Surface, TS: Total Surface, BPm: Bone Perimeter, BTh: Bone Thickness, Ctr: control.

Tiss-Zn²⁺ achieved higher bone perimeter (BPm) than that produced by TissHYD (**Table 10**).

Table 10. Statistical results of P values after data analysis. Bold letters indicate significance at P ≤ 0.05.

Ref	BS	OS	OS/TS	BPm	BTh
Ctr vs Tiss-Zn²⁺	0.04	0.04	0.90	0.41	0.00
Ctr vs Tiss-Ca²⁺	0.05	0.27	0.80	0.31	0.01
Ctr vs TissHYD	0.04	0.18	0.63	0.37	0.02

Abbreviations: BS: Bone Surface, OS: Osteoid Surface, TS: Total Surface, BPm: Bone Perimeter, BTh: Bone Thickness, Ctr: control.

For comparison in **Fig. 13** are shown bone sections stained by the von Kossa silver nitrate technique at six weeks of follow up, with no membrane -control (**Fig. 13A**)- and Tiss-Ca²⁺ membrane (**Fig. 13B**). Trabecular bone formation were observed along the margin of calvarial defect (arrow head), and within the defect. The pointers of **Fig. 13** (Mbr: membrane, NB: new bone and OB: old bone) show scattered bone islands, in

correspondence with new bone. The bone defect in the control group was found to be filled with connective tissue and a few immature bone trabeculae (**Fig. 13A**). Areas of trabecular bone formation could also be identified in the defects treated with either type of the membrane (**Figs. 12B, 13B**).

- 5 **Table 11.** Bone cells and blood vessels detected within the new bone formed in the region of interest (ROI) (Mean $\pm$ Standard Deviation SD).

Ref	Osteocytes/mm ²	Osteoblasts/mm ²	Blood Vessels/mm ²
TissHYD	2934.74 $\pm$ 1231.68	154.97 $\pm$ 51.66	11.66 $\pm$ 2.95
Tiss-Zn ²⁺	3359.91 $\pm$ 783.15	312.63 $\pm$ 92.41	14.77 $\pm$ 1.88
Tiss-Ca ²⁺	3200.29 $\pm$ 1450.04	307.16 $\pm$ 96.23	16.31 $\pm$ 3.67
Ctr	2796.25 $\pm$ 646.30	247.61 $\pm$ 66.36	16.58 $\pm$ 10.11

Abbreviations: Ctr: control.

Table 12. Statistical results of P values after data analysis with paired t-tests. Bold letters indicate significance at $P \leq 0.05$.

Ref	Osteocytes	Osteoblasts	Blood vessels
Ctr vs Tiss-Zn ²⁺	0.33	0.00	0.28
Ctr vs Tiss-Ca ²⁺	0.45	0.02	0.03
Ctr vs TissHYD	0.61	0.11	0.36

- 10 **Abbreviations:** Ctr: control.

Both Tiss-Zn²⁺ and Tiss-Ca²⁺ promoted higher number of osteoblasts than the control group. The number of osteoblasts was higher in subjects treated with Tiss-Zn²⁺ membranes than with unloaded membranes (**Table 12**). In some fields of all samples, osteoblasts were observed in the process of opposing bone directly on the membrane surface (**Figs. 14A and 14B**). Tiss-Ca²⁺ did not produce greater number of osteoblasts than the rest of the membranes but originated higher number of blood vessels than the control group (**Table 12**). Tiss-Ca²⁺ showed dense and neat collagen fibers that run parallel to the bone defect and membrane. The control group promoted lower amount of blood vessels than Tiss-Ca²⁺. Many large vessels could be detected in samples treated with Tiss-Zn²⁺ membranes (**Fig. 14A**). Small blood vessels were shown in close proximity to the new bone and the Tiss-Ca²⁺ biomaterial.

Images obtained with TB also permitted to observe that Tiss-Zn²⁺ and Tiss-Ca²⁺ membranes promoted the formation of bond matrix (**Fig. 14**) over the membrane, outside the surgical defect. No inflammatory cells or multinuclear giant cells were present at the interface with bone in animals treated with Tiss-Zn²⁺ (**Fig. 14A**).

5 In this experimental study, bone regeneration accounted was observed in all groups. At the end of the study the size of the defects was smaller than its original size. All bone defects showed mineralized bone surface within the region of interest (**Fig. 12A**), but Tiss-Zn²⁺, Tiss-Ca²⁺ and TissHYD attained significantly higher new formed bone (BS) in comparison to controls (**Tables 9 and 10**). The tissue pattern appeared composed by
10 membranes in close contact to the newly-formed bone and to osteoid tissue. The significantly increase of bone surface (BS), *i.e.*, mineralized bone matrix excluding osteoid and bond thickness (BTh) became associated with a generalized rise of osteoblasts promoted by all membranes when compared with the control group, especially when Tiss-Zn²⁺ membranes were used (tables 9 and 10). New bone was
15 observed directly in contact with the Tiss-Zn²⁺ membrane surfaces in the regions displaying successful bone conduction or (**Fig. 14A**). Newly formed bone was continuous from the defect margin without any invasion of the soft tissue. Continuously regenerated bone adhered to the Tiss-Zn²⁺ membrane forming bony bridging images (**Fig. 14B**). Multiple interconnected ossified trabeculae were shown at the region of
20 interest (**Fig. 12A**).

The Application of Tiss-Zn²⁺, Tiss-Ca²⁺ and TissHYD membranes induced significant changes in remodeling and structural indices of bone. This increase remodeling might result in the replacement of older, overly mature bone with younger and more resilient bone (Rubin et al., 2018). Osteoid or bone matrix that will be, but not yet, mineralized
25 showed higher surface than in the control group when membranes were used, typical of young bone (La Monaca et al., 2018).

Not only osteogenesis but enhanced biological activity was also observed after determining the amount of osteoblasts when Tiss-Zn²⁺ and Tiss-Ca²⁺ membranes were used (**Table 10**). Tiss-Zn²⁺ has been shown to enhance cell proliferation/wound healing
30 (Augustine et al., 2014). Formation of new bone indicates that membranes can induce osteoblasts to fill, or partially fill intracortical pores by nucleating clusters which induce their subsequent fusion to form amorphous calcium phosphate and ultimately apatite crystals, thus reactivating bone-lining cells to bone-forming osteoblasts (Vrahnas et al., 2018). Previous zinc complexation on tissues has been demonstrated to facilitate

phosphate group binding with biomimetic precipitation of Ca/P deposits and formation of hydroxyapatite nanocrystals. These phosphate groups, at the surface, have under-coordinated oxygens, which lead to reactive surfaces that will attract calcium ions from the media. This biomimetic deposition of Ca/P is considered as a coating method inspired by the natural process of biomineralization. Even more, phosphoproteins are thought to supply phosphate for mineralization, being capable of modulating crystal nucleation and growth, as well as binding to the collagenous network. Higher solubility of ZnO when in contact with acid substrates, as some acidic non collagenous proteins, could also account for the effective release of zinc ions, which stimulates protein phosphorylation, enhanced calcium deposition, and facilitated crystals precipitation. Osteoblasts stimulated with extracellular Ca^{2+} , and PO_4^{2-} increased bone morphogenetic protein-2 mRNA expression (Shimauchi et al., 2013; Tada et al., 2010). Moreover, the pore connectivity described in these membranes might influence the possibility that a greater number of osteoblasts can penetrate the porous structure (Guarnieri et al., 2018). In addition, an ingrowth of micro vessels was also found nearby the membrane (**Fig. 14A**) when Tiss- Zn^{2+} membranes were used, contributing to the integration of the biomaterial in the tissue (Turri and Dahlin, 2015).

Present findings provide further evidence that these membranes act as bioactive modulator for signals communicated to the underlying defects. These results provide evidence that membranes are useful and provide advantageous for bone tissue regeneration.

5. ANTIBACTERIAL EFFECTS OF TissHYD, Tiss- Zn^{2+} , Tiss- Ca^{2+} AND Tiss-DOX.

Hydroxylapatite (HAp) discs with a 7 mm of diameter and a thickness 1.8 mm (Clarkson Chromatography Products, Williamsport, PA, USA) were bonded to four types of membranes: TissHYD, Tiss- Ca^{2+} (loaded with $1.1 \mu\text{g}(\text{Ca}^{2+})/\text{mg}$ Tiss), Tiss- Zn^{2+} (loaded with $1.1 \mu\text{g}(\text{Zn}^{2+})/\text{mg}$ Tiss), and Tiss-DOX (loaded with $0.8 \text{ mg}(\text{DOX})/\text{mg}$ Tiss). Naked HAp discs and HAp discs covered with a PTFE membrane were used as a control. All the specimens were used to develop a multispecies oral biofilm. Reference strains *Streptococcus oralis* CECT 907T, *Veillonella parvula* NCTC 11810, *Actinomyces naeslundii* ATCC 19039, *Fusobacterium nucleatum* DMSZ 20482, *Aggregatibacter actinomycetemcomitans* DSMZ 8324 and *Porphyromonas gingivalis* ATCC 33277 were used. In brief, pure cultures of each bacterium were grown anaerobically in a protein-rich medium containing brain-heart infusion. HAp discs control and those covered with the different membranes were then placed in the wells of a 24-well tissue culture plate. Each well was then inoculated with 1.5 mL mixed

bacteria suspension prepared, and incubated in anaerobic conditions (10% H₂, 10% CO₂, and balance N₂) at 37°C for 12, 24, 48 and 72 h. Plates containing only culture medium will be also incubated to check for sterility.

5 Biofilms from 12 to 72 hours of evolution were observed by Scanning Electron Microscopy (SEM). For this analysis, the specimens were fixed in a solution at 4% paraformaldehyde and 2.5% glutaraldehyde for 4h at 4°C. After that, specimens were critical point dried, sputter-coated with gold and analysed.

10 For quantitative evaluation of bacteria growth biofilm DNA, from 12, 24, 48 and 72 hours incubation was isolated using a commercial kit (MolYsis Complete5; Molzym GmgH & CoKG, Bremen, Germany), following manufacturer's instructions (the protocol for bacterial DNA extraction was followed from step 6, avoiding preliminary steps). The hydrolysis probe 5' nuclease assay PCR method was used for detecting and quantifying the bacterial DNA. Primers and probes were obtained by Life Technologies Invitrogen (Carlsbad, CA, USA), Applied Biosystems (Carlsbad, CA, USA) and Roche
15 (Roche Diagnostic GmbH; Mannheim, Germany) and were targeted against 16S rRNA gene. The quantitative Polymerase Chain Reaction (qPCR) amplification was performed in a total reaction mixture volume of 10 µL. Analyses were performed with a LightCycler® 480 II thermocycler (Roche). The plates used in the study was FramStar 480 of natural frame and white wells (4titude; The North Barn; Damphurst Lane, UK),
20 sealed by QPCR Adhesive Clear Seals (4titude). Each DNA sample was analyzed in duplicate. Quantification cycle (C_q) was determined using the provided software package (LC 480 Software 1.5; Roche). Quantification of cells by qPCR was based on standard curves. The correlation between C_q values and CFU mL⁻¹ were automatically generated through the software (LC 480 Software 1.5; Roche).

25 Shapiro–Wilk goodness-of-fit tests and distribution of data were used to assess normality. Data were expressed as means and Standard Deviation (SD). To compare the effects of the material surface at different exposure times on CFU mL⁻¹, analysis of variance and post-hoc testing with T3 Dunett's correction was used. Results were considered statistically significant at p<0.05.

30 FESEM images of different biofilms formation are shown in the **Fig. 15**. After 24 h, high quantities of bacteria were observed on all the discs forming a thick layer of bacteria, with initial characteristics of a structured biofilm. Except for Tiss-Dox discs that showed absent or few isolated bacteria, onto the surfaces. Microbial communities interspersed

with channels could be observed, suggesting that bacteria may have reached the exponential phase of growth. No significant differences among the specimen discs were found in regards to the structure of the biofilm, except with biofilms on Tiss-DOX discs, which lacked an organized structure.

- 5 Bacterial counts (CFU mL⁻¹) for the six species at 72 h incubation time in the tested specimens are shown in **Fig. 16**. With time, the dynamics of bacterial growth were similar independent from the specimen. Biofilms on HAp discs coated with Tiss-DOX reached the lowest numbers of bacteria, when is compared with the rest of the groups (p<0.01).

10 **6. TissHYD DOPING WITH SiO₂ NANOPARTICLES (NPS-SiO₂)**

Silicon dioxide (SiO₂) is able to improve not only bioactivity of materials but also cell adhesion and proliferation on artificial tissues, facilitating osteogenic cells differentiation. SiO₂ is considered to be osteoinductive and a catalyst for bone formation. Therefore, in order to improve the bioactivity of membranes they were doped
15 with SiO₂ nanoparticles (NPs-SiO₂) by two different ways:

- 1) 1g of NPs-SiO₂ was added to the optimum electrospinning scaling solution (see section 6), and the solution was prepared as follows: 1.000g of NPs-SiO₂ (1.219 % wt) was dispersed in 70.000 g of DMF (85.316% wt) by 20 min of sonication, then 5.000g of (MA)₅-co-(HEA)₅ (6.094 % wt), and 6.000 g of (MMA)₃-co-(HEMA)₂ (7.313% wt) were dissolved in the NPs-SiO₂/DMF suspension. When
20 the copolymers were completely dissolved, 0.048 g of hydrochloric acid (HCl) (0.058% wt) were added to the solution. Then by using the parameters of the **Table 7.**, the solution was processed by electrospinning, and a nonwoven nanofibers mat doped whit NPs-SiO₂ (TissSi-OH) was obtained: The
25 mechanical properties of the membranes were not affected by the incorporation of NPs-SiO₂. Then, in order to introduce carboxyl groups (COOH) on the fibers surface, a partial hydrolysis of ester groups: R-COCH₃ and RCOCH₂CH₂OH of TissSi-OH was carried out to obtain TissHYDSi. The hydrolysis was done by introducing both membranes in a sodium carbonate (333 mM) pH=12.50
30 solution during 30min. Then the membranes were washed 3 times with distilled water and dry at room temperature. The number of accessible COOH groups calculated by TBO method was 660±50μmol/g.
- 2) TissHYD was soaked with a suspension of 10-20nm of SiO₂ nanoparticles (SiO₂-NPs), and then the water was evaporated in a vacuum at constant

temperature: by this way the membrane was loaded with 0.06 mg of SiO₂-NPs /mgTiss.

Then the membranes doped with SiO₂-NPs by procedure 1) and 2) (TissHYDSi) were functionalize with zinc, calcium and DOX through the following protocols:

5 **6.1. Functionalization of TissHYDSi with Zn²⁺ (TissSi-Zn²⁺) and Ca²⁺ (TissSi-Ca²⁺).**

The ability of carboxyl groups to complex divalent cations was used to functionalise TissHYDSi with Zn²⁺ and Ca²⁺. TissHYDSi was soaked with a Zn²⁺ and Ca²⁺ solution, and then the water was evaporated in a vacuum at constant temperature: by this way TissHYDSi was loaded with 1.1 µg/mg, of Ca²⁺ and Zn²⁺.

10 **6.2. Functionalization of TissHYDSi with doxycycline (TissSi-DOX).**

Doxycycline (DOX) was bound non-covalently into TissHYDSi by acid-base interactions between amine groups of DOX and carboxyl groups of TissHYDSi as well as by hydrogen bonds between the hydroxyl groups of membrane and amine groups of DOX. TissHYDSi was soaked with a DOX solution, and then the water was evaporated in a vacuum at constant temperature: by this way TissHYDSi was loaded with 0.8 mg of DOX/mg Tiss.

15 **7. ACELLULAR STATIC IN VITRO BIOACTIVITY TEST OF TissHYDSi, TissSi-Zn²⁺, TissSi-Ca²⁺ AND TissSi-DOX.**

To probe for the effect of silicon dioxide inclusion in membranes' bioactivity, the analysis proposed by Kokubo (ISO 23317:2012. Implants for surgery In vitro evaluation for apatite-forming ability of implant materials) was performed as detailed previously in section number 3.1.

Results from FESEM images of Tiss-SiO₂-COOH, Tiss-SiO₂-Ca²⁺, Tiss-SiO₂-Zn²⁺ and Tiss-SiO₂-DOX carried out by procedure 1) after 7 days of immersion are presented in **Fig. 17**. Bioactivity of tissues was demonstrated in all cases. The inclusion of silicon dioxide on sample tissues has drastically augmented the appearance of mineral deposits in all tested surfaces (**Fig. 11**: without SiO₂ and **17**: with SiO₂).

Rounded deposits were abundant and uniformly distributed onto nanofibers in all cases: similar results were obtained with the materials doped with SiO₂ by the procedure 2).

SiO₂-doped membranes have shown to increase the bioactivity through an augmented mineralisation.

CLAIMS

1. A hydrophilic nonwoven nanofibers membrane, characterised in that it comprises a blend of

- 5
- a first copolymer of (MA)₃-co-(HEA)₂ with statistical topology in a weight percent between 35% and 65%; and
 - a second copolymer of (MMA)₁-co-(HEMA)₁ in weight percent between 35% and 65%.

10 2. The hydrophilic nonwoven nanofibers membrane according to claim 1, characterised in that it comprises a blend of

- a first copolymer of (MA)₃-co-(HEA)₂ with statistical topology in a 50% by weight; and
- a second copolymer of (MMA)₁-co-(HEMA)₁ in a 50% by weight.

15 3. The hydrophilic nonwoven nanofibers membrane according to any of claims 1 or 2, characterised in that the first copolymer of (MA)₃-co-(HEA)₂ has a molecular weight between 50000 Da and $3 \cdot 10^6$ Da, preferably above 80000 Da.

20 4. The hydrophilic nonwoven nanofibers membrane according to claim 3, characterised in that the first copolymer of (MA)₃-co-(HEA)₂ has molecular weight between $1 \cdot 10^6$ Da and $3 \cdot 10^6$ Da.

25 5. The hydrophilic nonwoven nanofibers membrane according to any of claims 1 to 4, characterised in that the second copolymer of (MMA)₁-co-(HEMA)₁ has molecular weight between 50000 Da and $1 \cdot 10^6$ Da.

30 6. The hydrophilic nonwoven nanofibers membrane according to any of claims 1 to 5, characterised in that it further comprises SiO₂ nanoparticles, wherein said SiO₂ nanoparticles are homogenously dispersed in the membrane and/or physically adsorbed on the surface of the membrane, in a weight percent between 0.1 % and 60 % with respect to the final weight of the membrane.

35 7. A process of preparation of the hydrophilic nonwoven nanofibers membrane according to any of claims 1 to 5, characterised in that it comprises the following steps:

- a) synthesis of the first copolymer of $(MA)_3$ -co- $(HEA)_2$ by Metal-Catalysed Living Radical Polymerization using a metal catalytic system;
- b) synthesis of the second copolymer of $(MMA)_1$ -co- $(HEMA)_1$ by reverse Atom transfer radical polymerization using a metal catalytic system;
- 5 c) preparation of a nanofibers membrane comprising a blend, said blend comprising the first copolymer obtained in step (a) and the second copolymer obtained in step (b) by electrospinning, and
- d) heat treatment of the nanofibers membrane obtained in step (c), wherein the heat treatment is applied in the form of hot water at a temperature range
- 10 between 30°C and 80°C and wherein the nanofibers membrane obtained in step (c) is kept tensioned by means of a frame.
8. The process according to claim 7, wherein the metal catalytic system of step (a) and step (b) is a copper amine complex.
- 15 9. The process according to claim 7, wherein the metal of the metal catalytic system of step (a) and/or step (b) comprises a transition metal or a mixture of transition metals in different oxidation states.
- 20 10. The process according to claim 9, wherein the metal of the metal catalytic system of step (a) and step (b) is independently selected from the list consisting of Cu, Fe, Co, Ni, Ru, Pt, Rh, Re, Cr and Mo.
- 25 11. The process according to any of claims 7, 9 or 10, wherein the ligand of the metal catalytic system of step (a) and/or step (b) is a multidentate aliphatic amine.
12. The process according to any of claims 7, 9 to 11, wherein the ligand of the metal catalytic system of step (a) and step (b) is independently selected from the list consisting of N,N,N',N'',N''-Pentamethyldiethylenetriamine (PMDETA), tris(2-pyridylmethyl)amine, tris[2-(dimethylamino)ethyl]amine, 2,2'-Bipyridyl, N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine and 1,1,4,7,10,10-hexamethyltriethylenetetramine.
- 30 13. The process according to any of claims 7 to 12, wherein the initiator of the metal catalytic system of step (a) is independently selected from the list consisting of dodecyl 2-bromoisobutyrate, ethyl α -bromoisobutyrate, octadecyl 2-
- 35

bromoisobutyrate, methyl α -bromoisobutyrate, methyl 3-bromopropionate, tert-Butyl 3-bromopropionate, ethyl 2-bromopropionate

14. The process according to any of claims 7 to 12, wherein the initiator of the metal catalytic system of step (b) is independently selected from the list consisting of 1,1'-azobis(cyclohexanecarbonitrile) (ACHN), 2,2'-azobis (2-methylpropionamidine) 2,2'-dihydrochloride (AAPH), 4,4'-azobis(4-cyanovaleric acid) (ACVA), tert-butyl hydroperoxide, cumene hydroperoxide, 2,5-di(tert-butylperoxide)-2,5-dimethyl-3-hexyne, dicumyl peroxide and 2,5-bis(tert-butylperoxide)-2,5-dimethylhexane.

10

15. The process according to any of claims 6 to 14, wherein the solvent used with the metal catalytic system of step (a) and step (b) is independently selected from the list consisting of acetone, dimethylformamide, poly(ethylene glycol), dimethyl sulfoxide, 1,4-dioxane, ethanol, propanol, hexane, water, carbon dioxide, ionic liquid, and a combination thereof.

15

16. The process according to any of claims 6 to 14, wherein no solvent is used with the metal catalytic system of step (a) and step (b).

17. The process according to any of claims 6 to 16, wherein the solvent used in step (c) is dimethylformamide.

20

18. The process according to any of claims 6 to 17, wherein the weight percent of the solvent used in step (c) is ranging between 20% and 98%.

25

19. A process of preparation of the hydrophilic nonwoven nanofibers membrane according to claim 6, wherein said SiO₂ nanoparticles are homogenously dispersed in the membrane, characterised in that it comprises all steps of the process according to any of claims 7 to 18 and wherein the blend of step (c) comprises SiO₂ nanoparticles.

30

20. A process of preparation of the hydrophilic nonwoven nanofibers membrane according to claim 6, wherein said SiO₂ nanoparticles are physically adsorbed on the surface of the membrane characterised in that it comprises all steps of the process according to any of claims 7 to 19 and a further step (e) of soaking the membrane obtained in step (d) in a suspension of SiO₂ nanoparticles and evaporate the solvent.

35

21. A hydrolysed hydrophilic nonwoven nanofibers membrane characterised in that it comprises the hydrophilic nonwoven nanofibers membrane according to any of claims 1 to 6 comprising carboxyl groups, wherein the concentration of carboxyl groups in the membrane is ranging between 20 $\mu\text{mol/g}$ of the membrane and 3000 $\mu\text{mol/g}$ of the membrane.

22. The hydrolysed hydrophilic nonwoven nanofibers membrane according to claim 21, characterised in that it is functionalised with a divalent cation selected from Zn^{+2} , Ca^{+2} , Mg^{+2} and Sr^{+2} , an antibacterial agent and/or any of the combinations thereof.

23. The hydrolysed hydrophilic nonwoven nanofibers membrane according to claim 21, characterised in that the hydrophilic nonwoven nanofibers membrane is functionalised with Zn^{+2} , Ca^{+2} and doxycycline.

24. A non-resorbable membrane for promoting bone regeneration characterised in that it comprises the hydrophilic nonwoven nanofibers membrane according to any of claims 1 to 6 or the hydrolysed hydrophilic nonwoven nanofibers membrane according to claims 21 to 23.

25. A non-resorbable periodontal membrane characterised in that it comprises the hydrophilic nonwoven nanofibers membrane according to any of claims 1 to 6 or the hydrolysed hydrophilic nonwoven nanofibers membrane according to claims 21 to 23.

26. A coating for an implant characterised in that it comprises the hydrophilic nonwoven nanofibers membrane according to any of claims 1 to 6 or the hydrolysed hydrophilic nonwoven nanofibers membrane according to claims 21 to 23.

FIG. 1

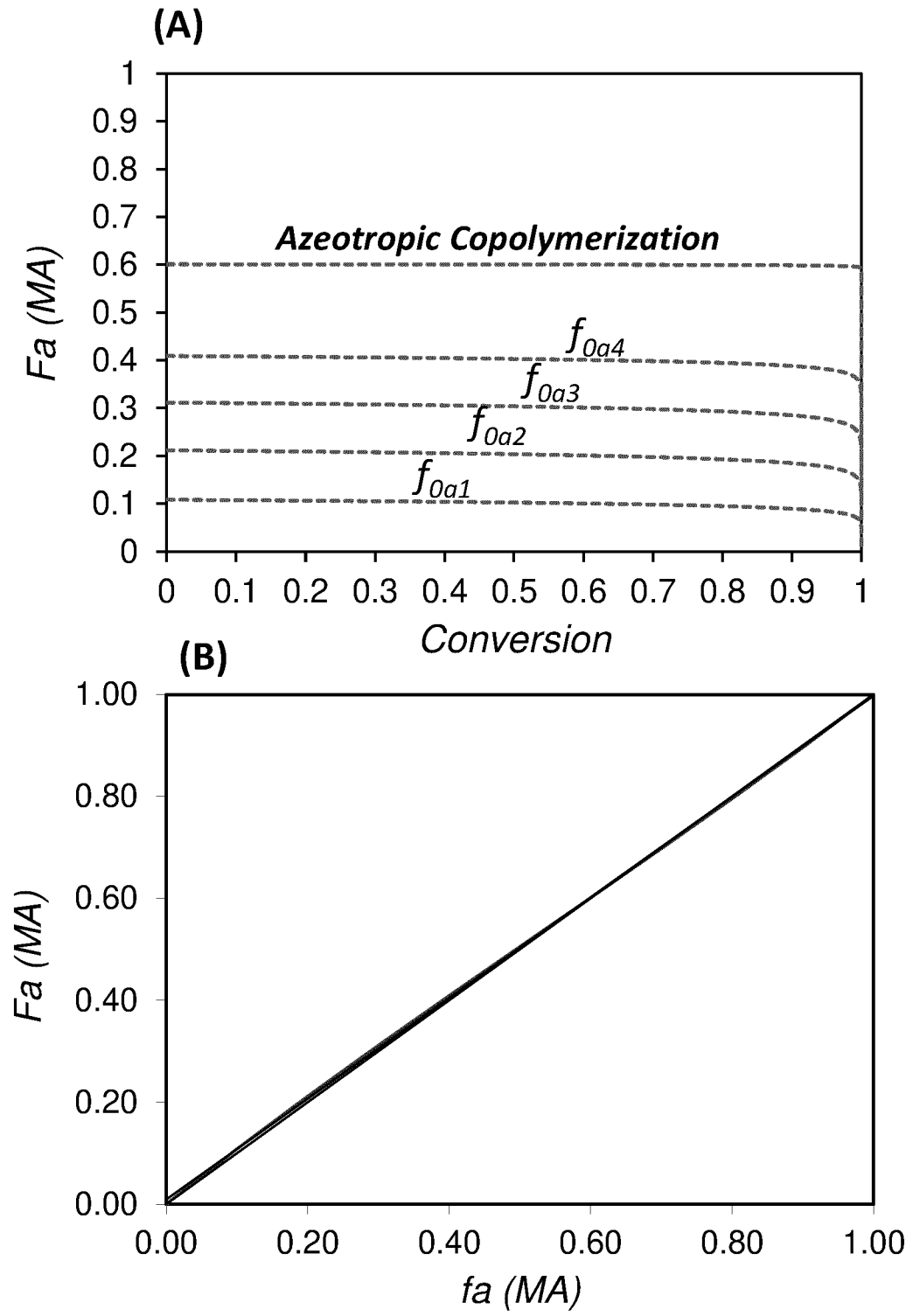


FIG. 2

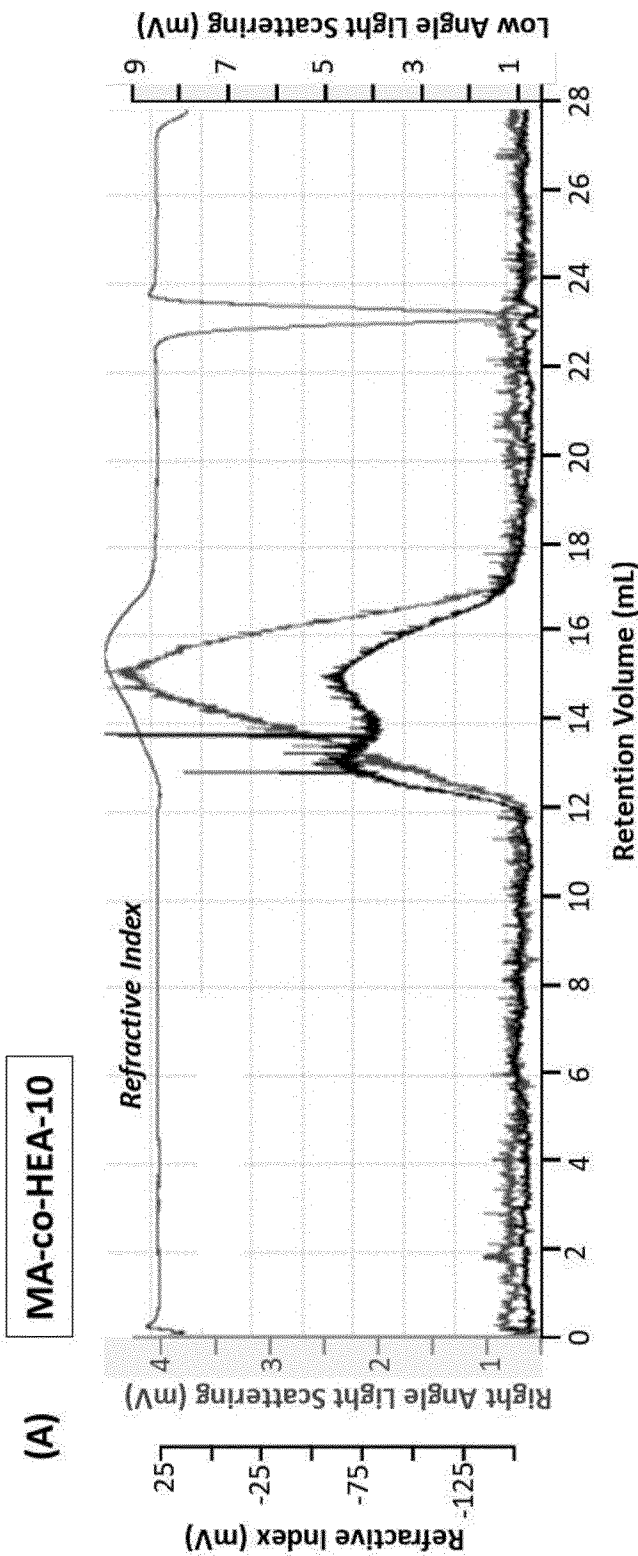


FIG. 2 cont.

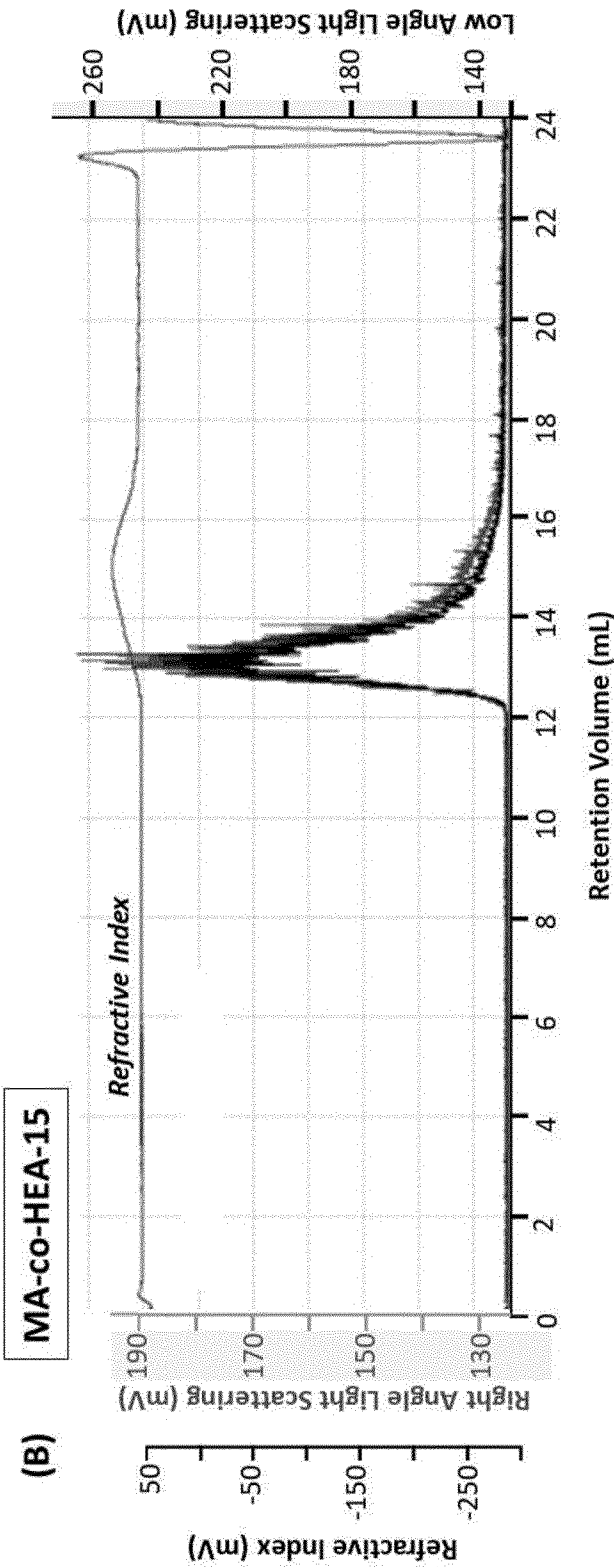


FIG. 2 cont.

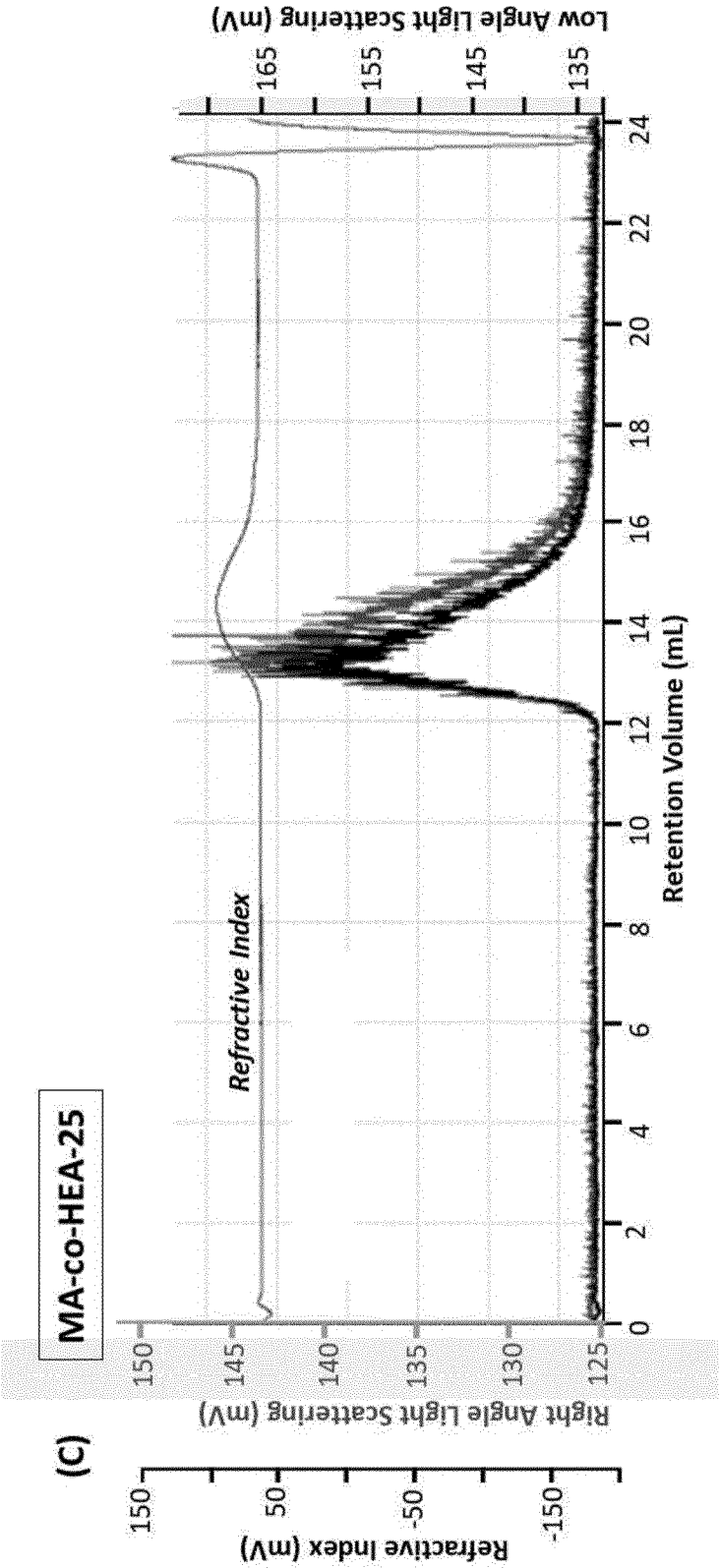


FIG. 2 cont

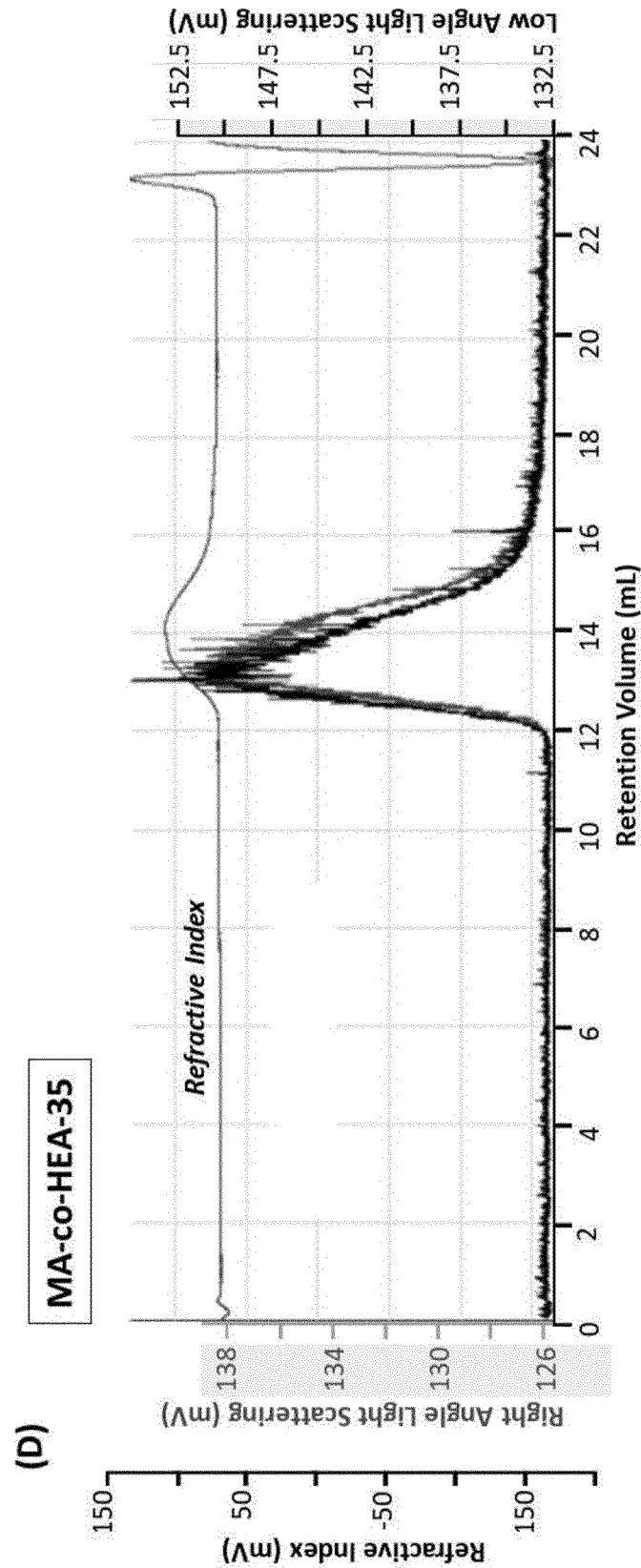


FIG. 2 cont

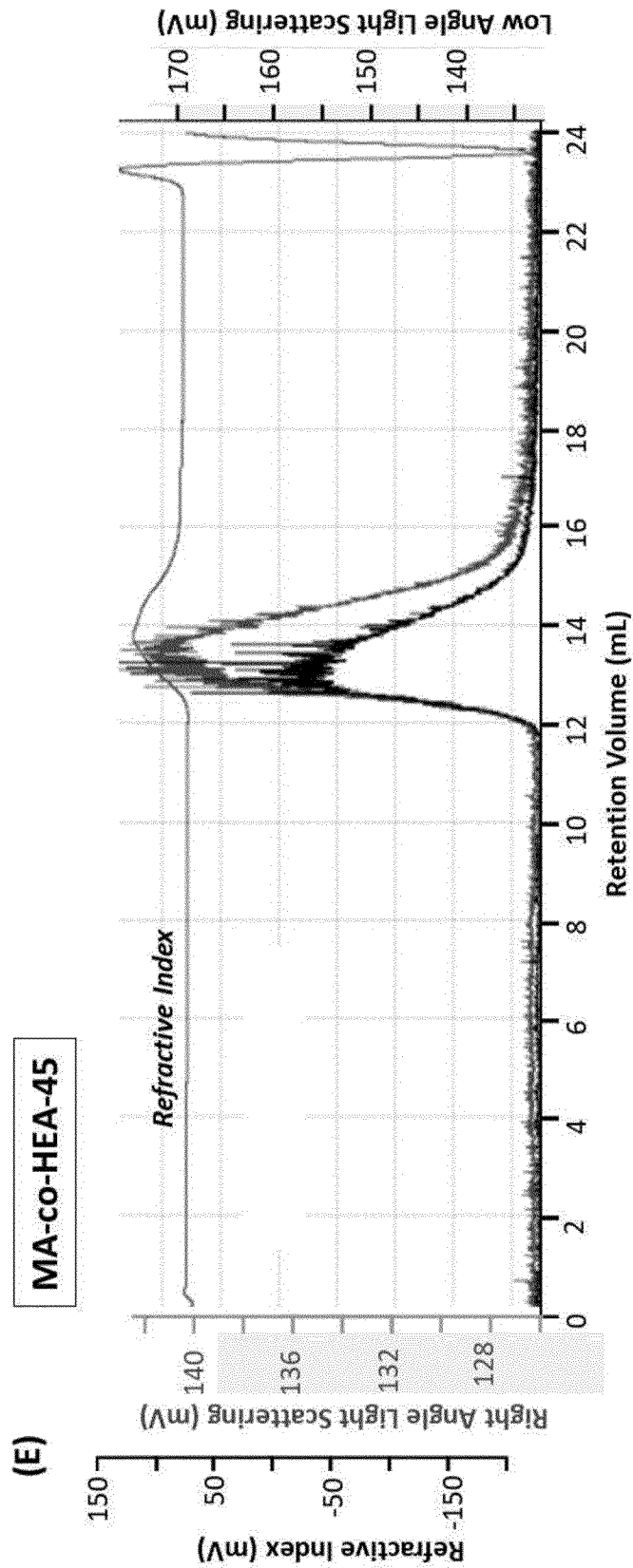


FIG. 3.

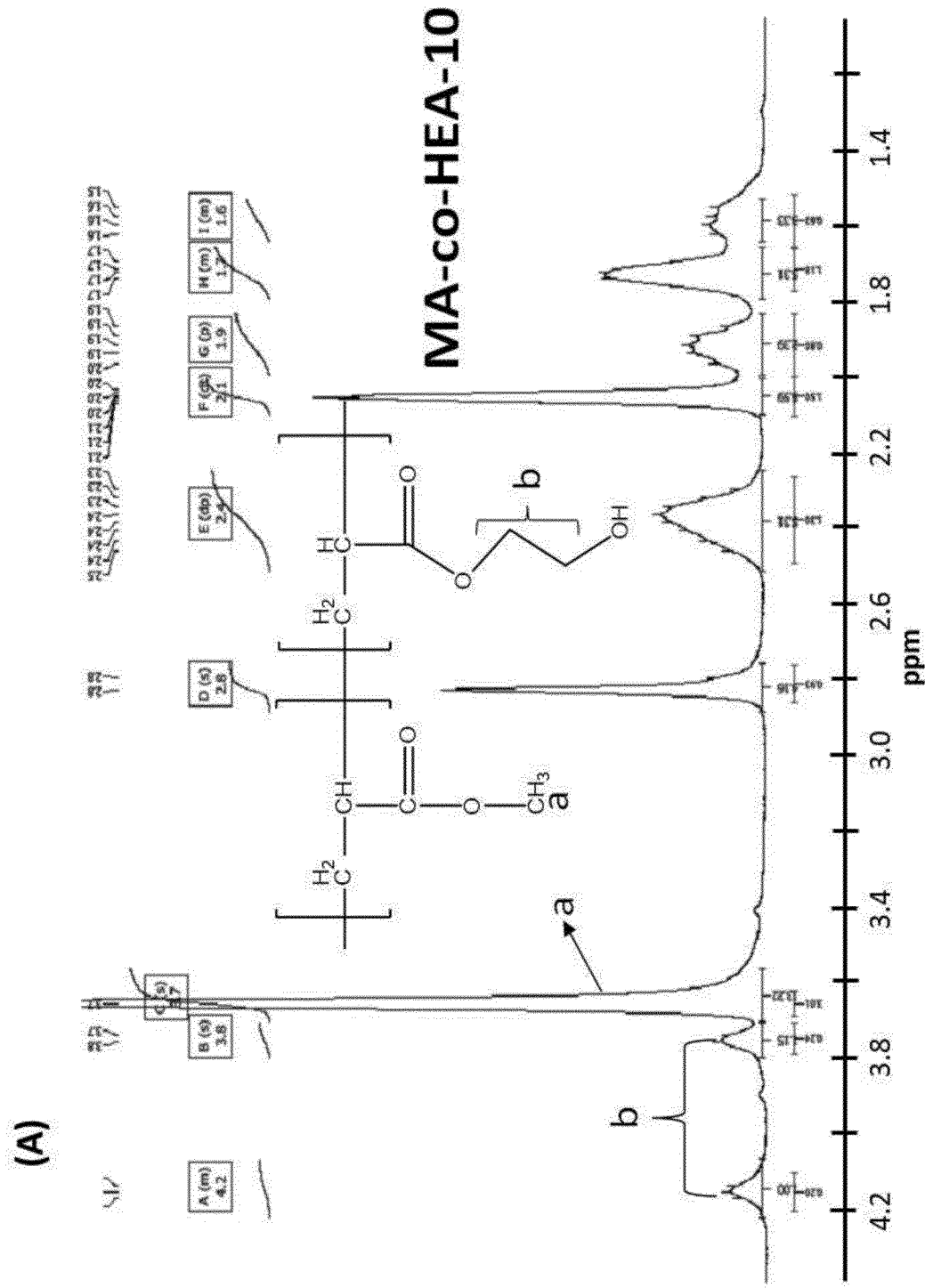


FIG. 3. Cont.

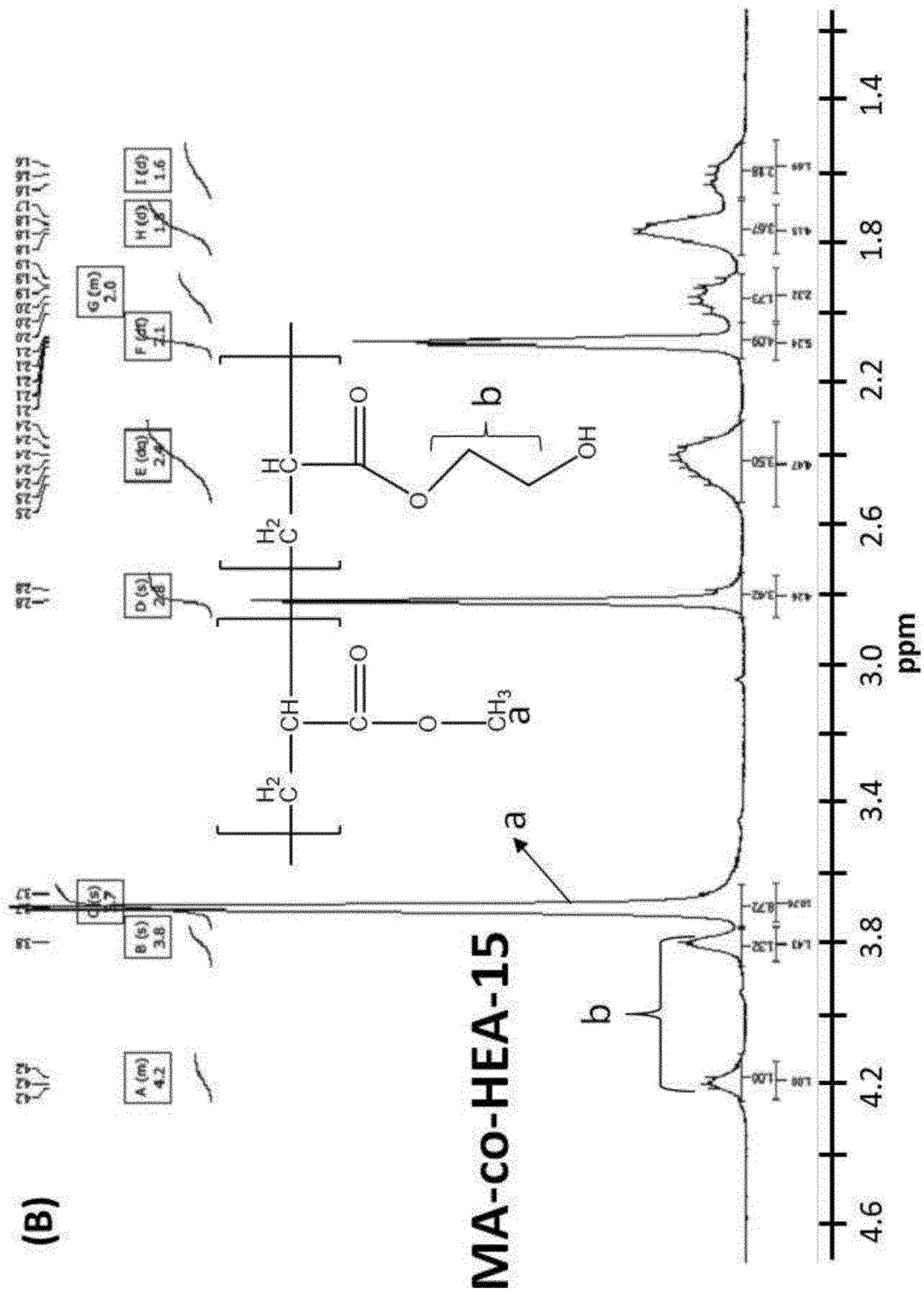


FIG. 3. Cont.

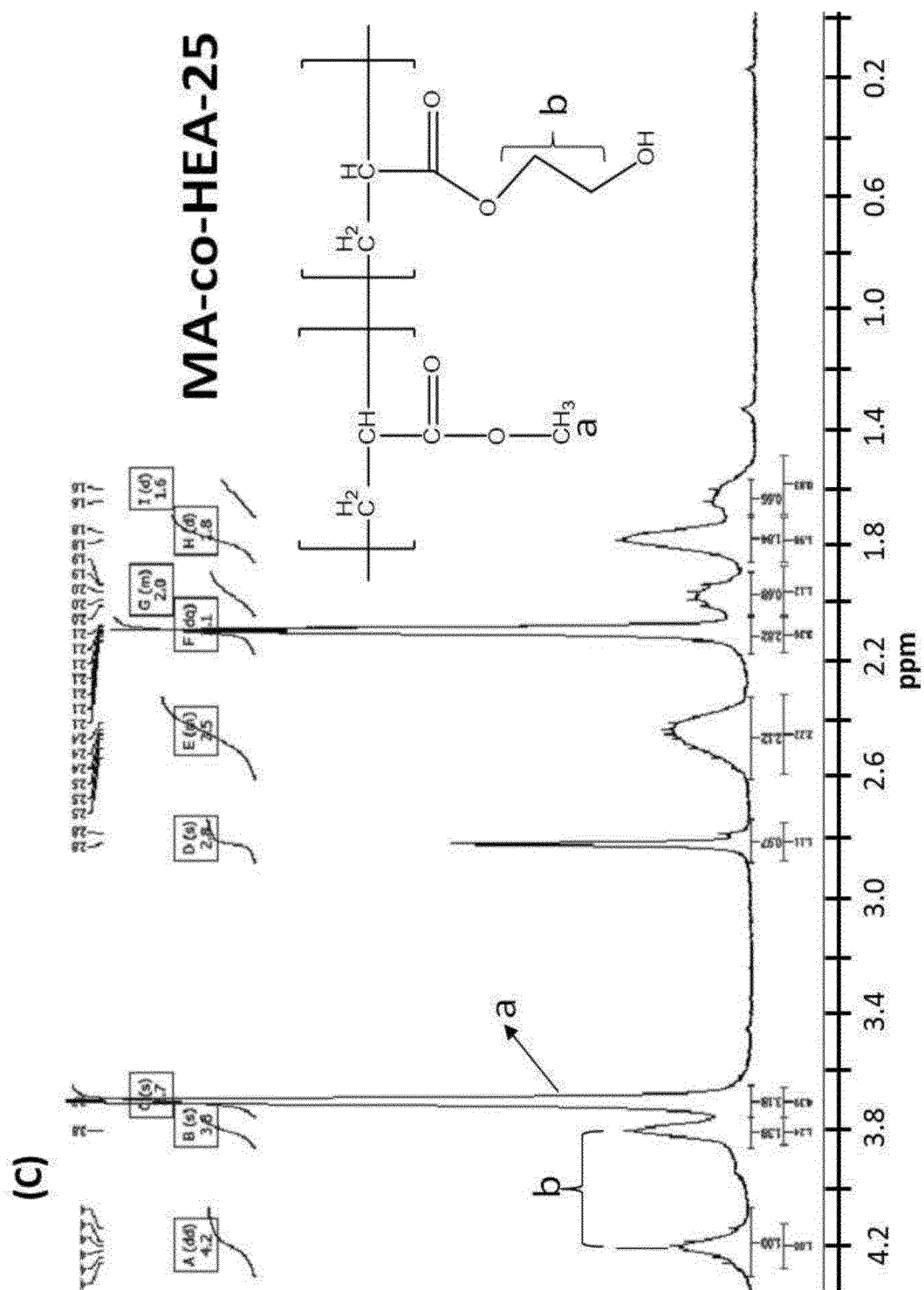


FIG. 3. Cont.

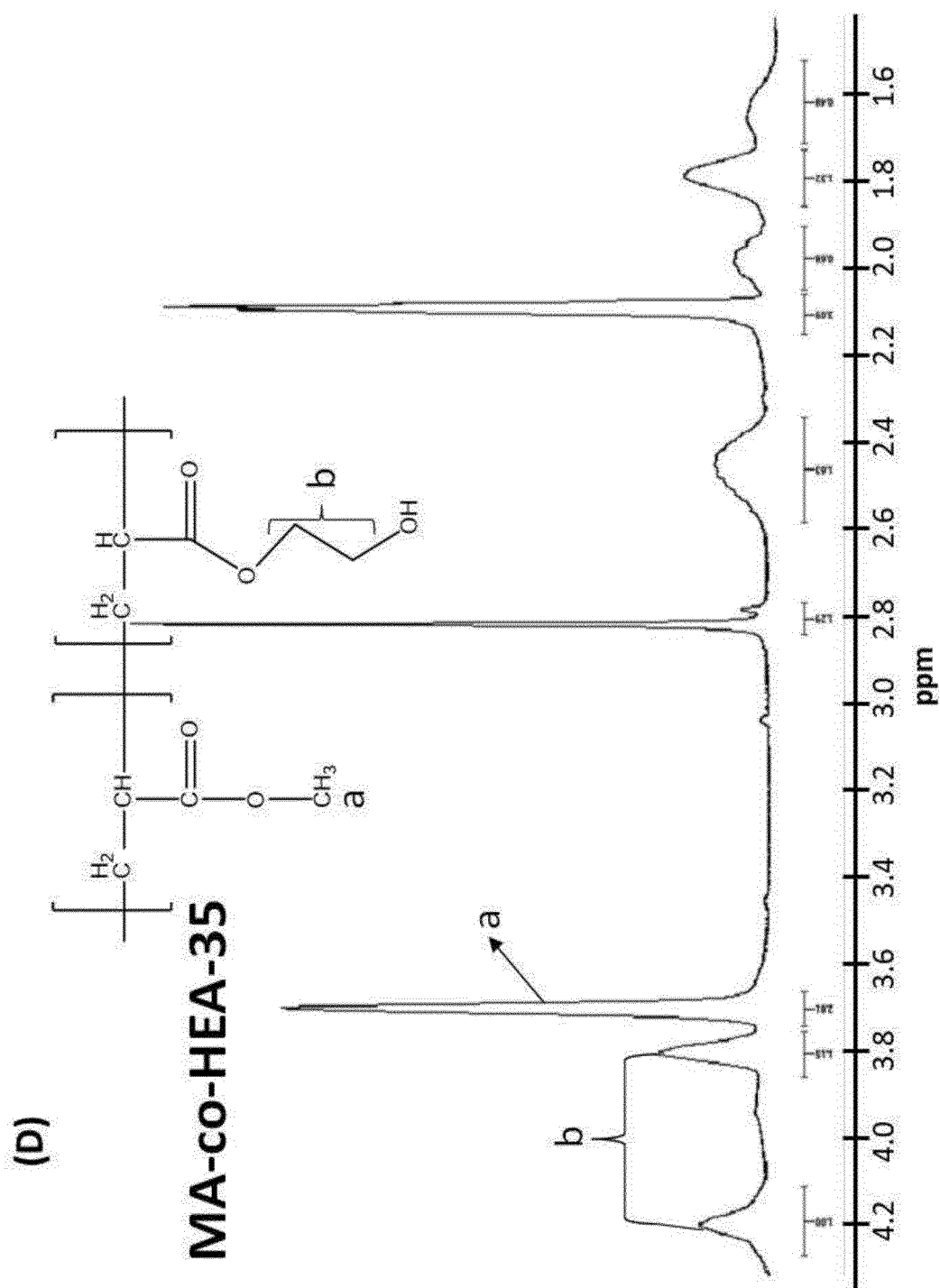


FIG. 4.

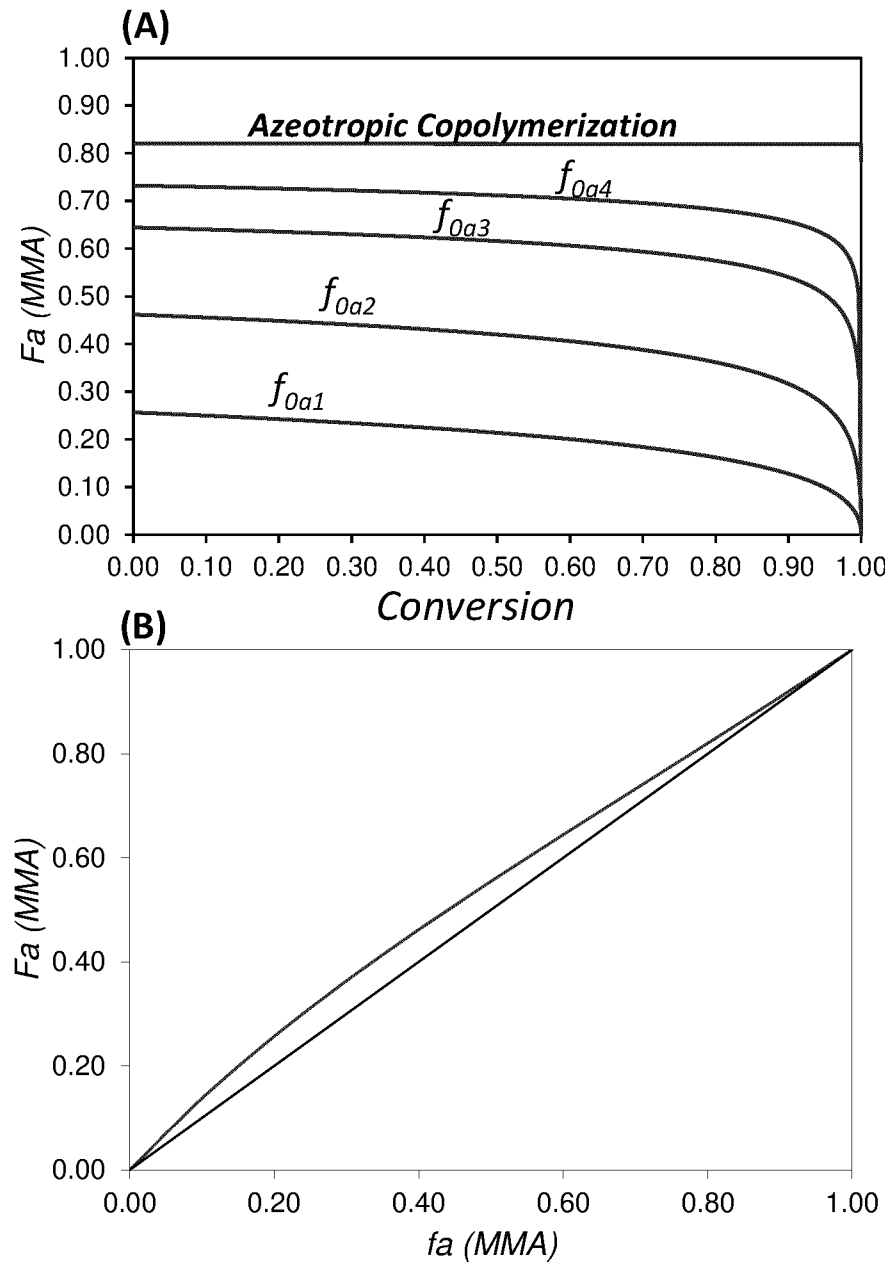


FIG. 5.

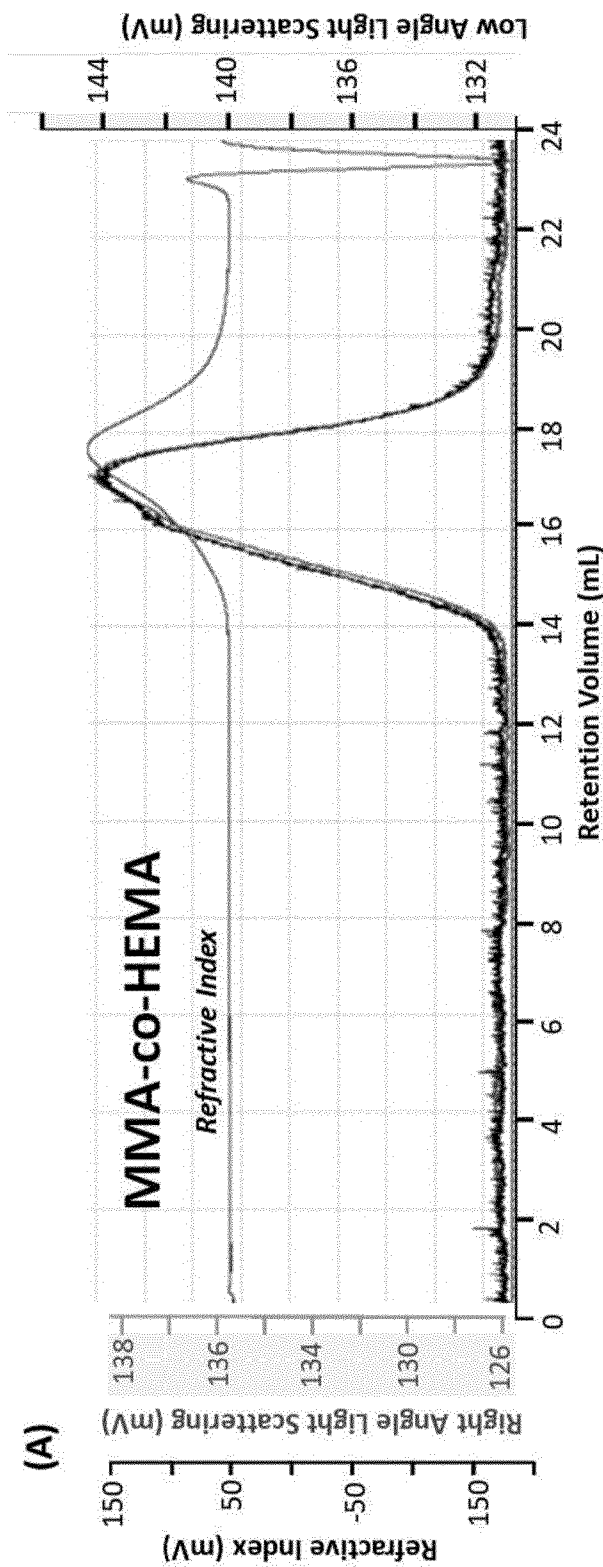


FIG. 5. Cont.

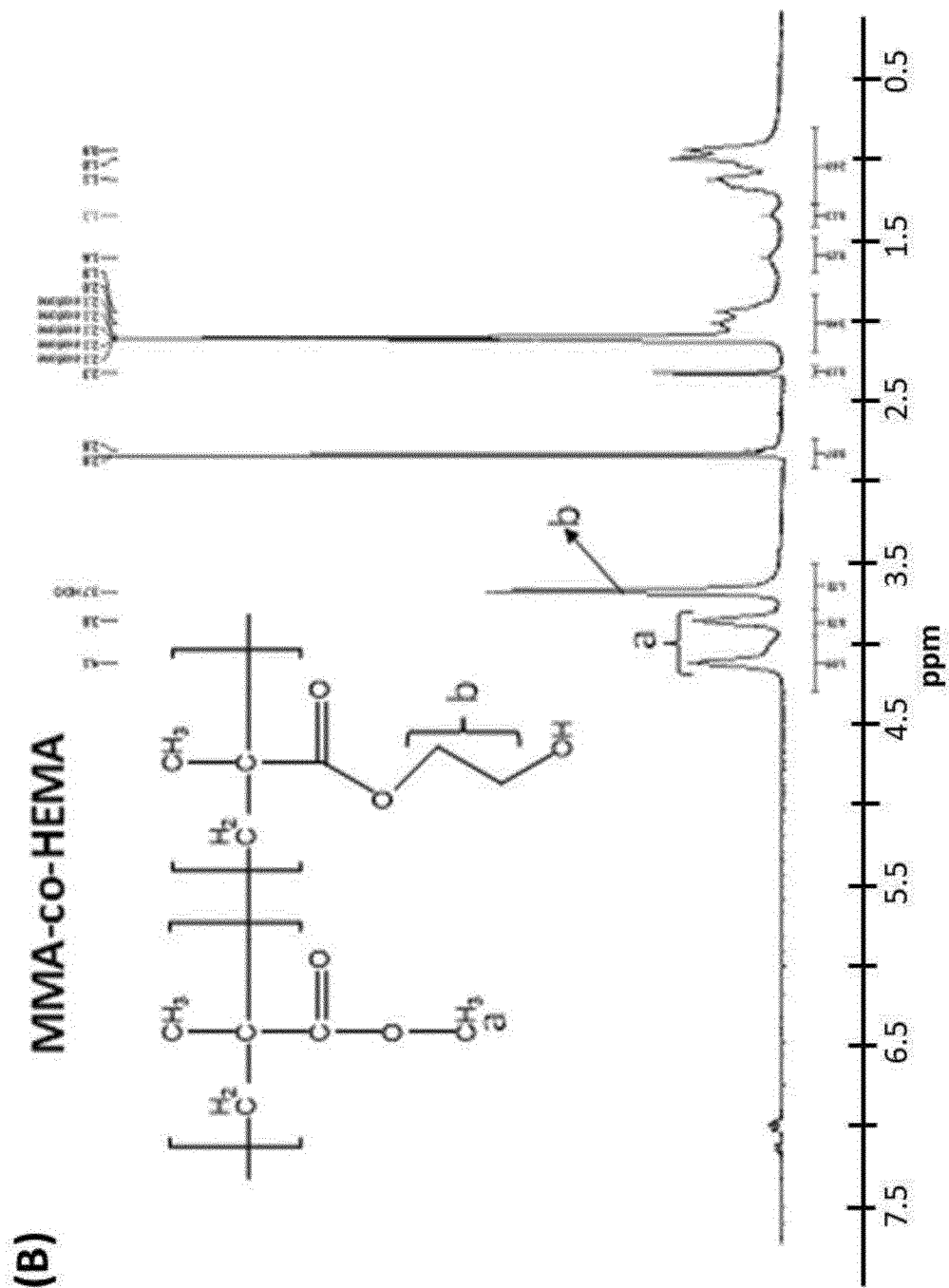


FIG. 6.

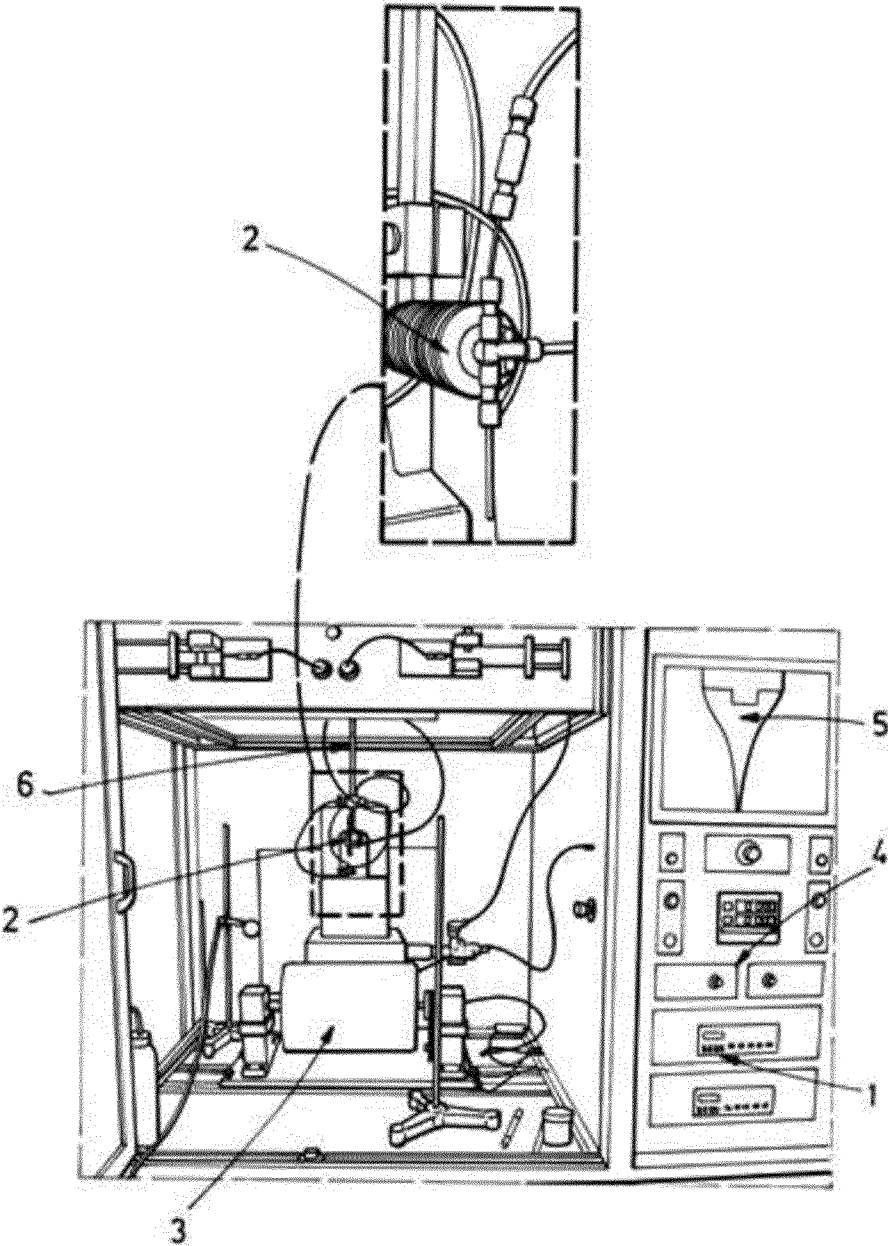


FIG. 7.

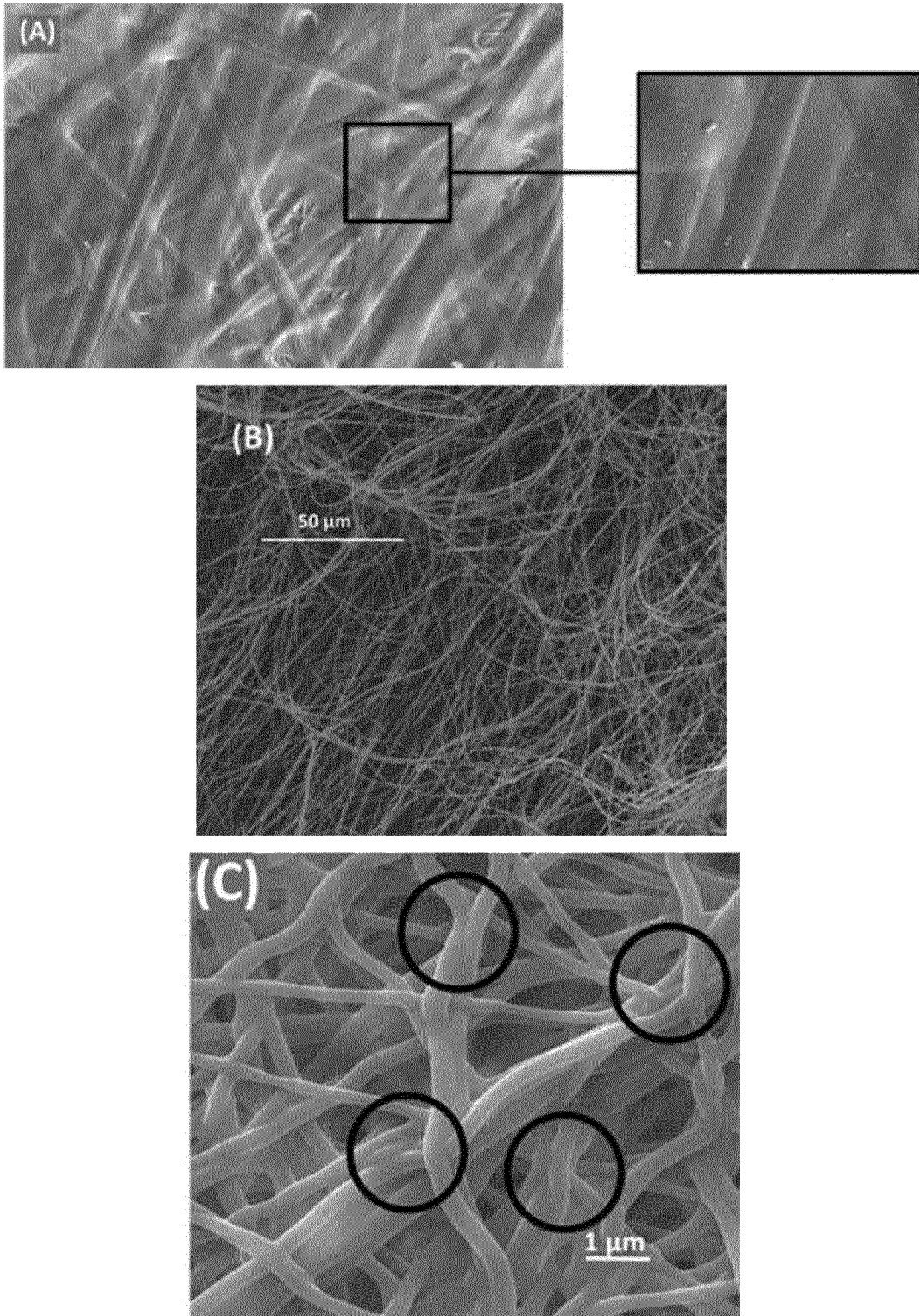


FIG. 7. Cont.

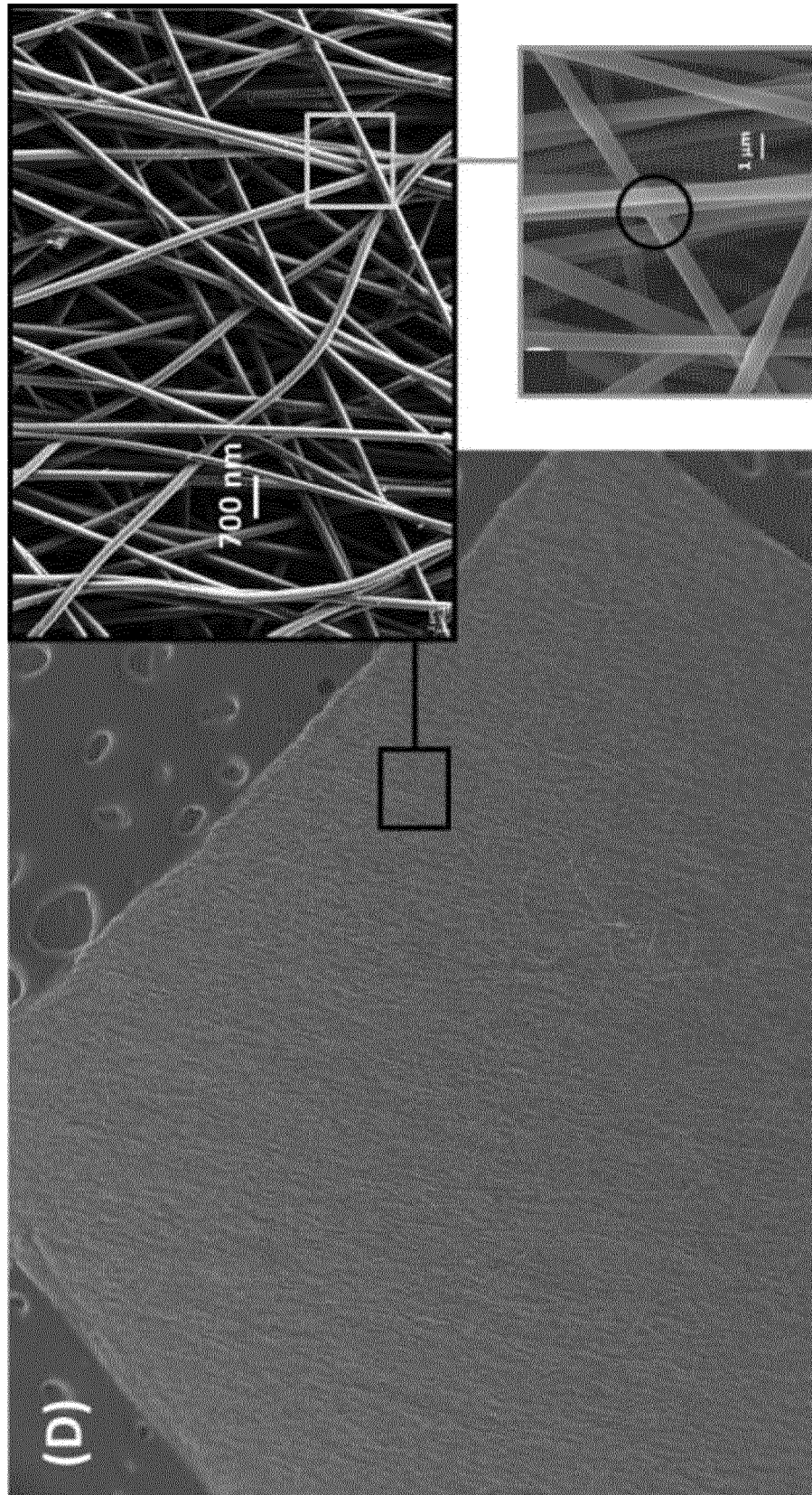


FIG. 7. Cont.

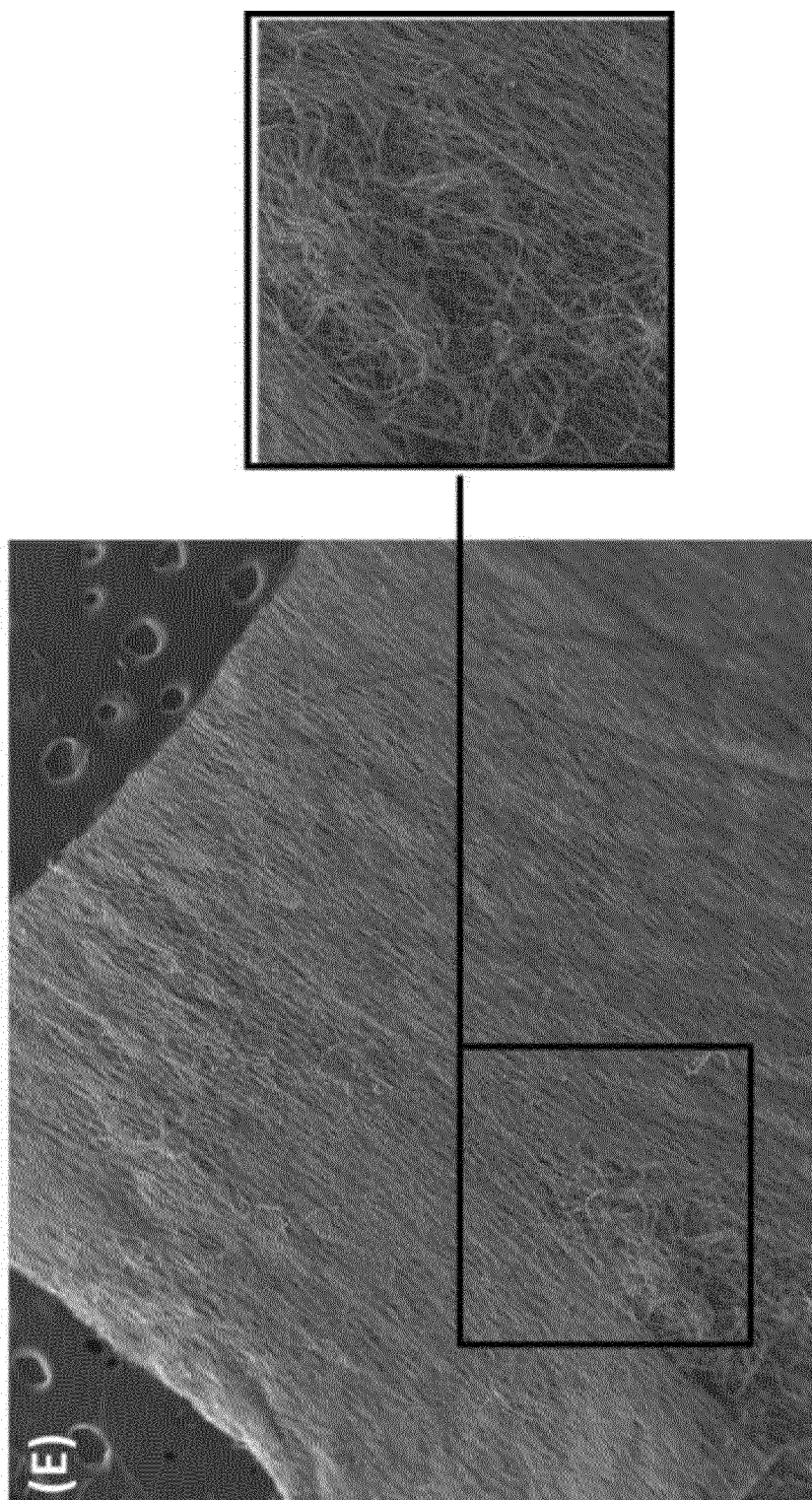


FIG. 8.

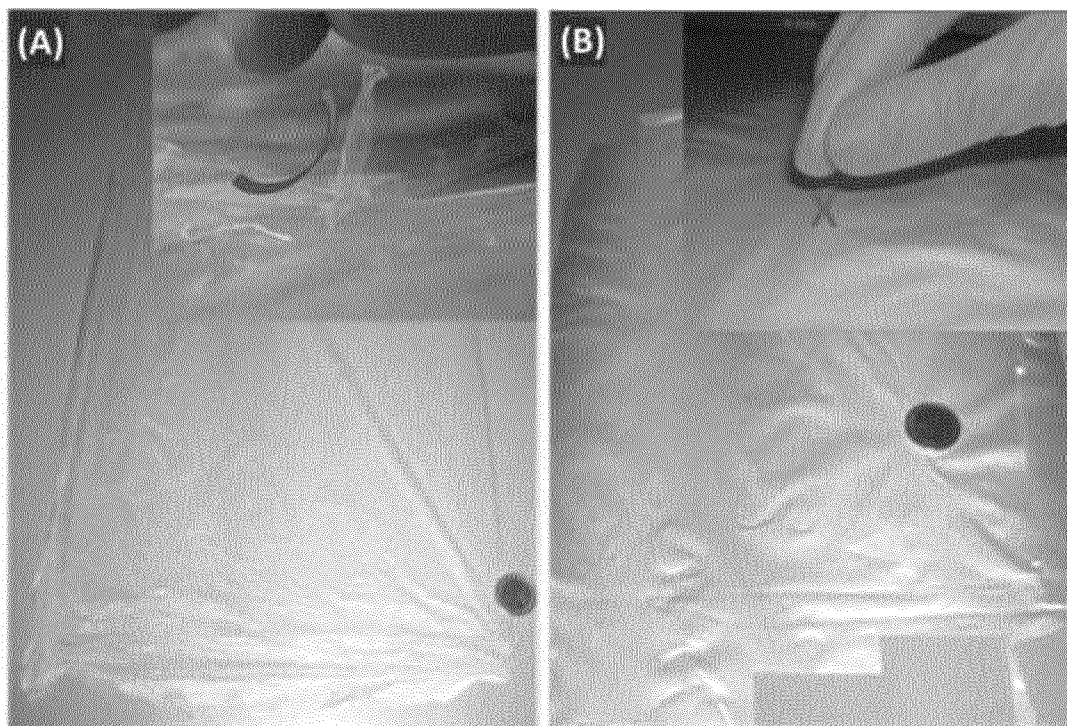


FIG. 9.

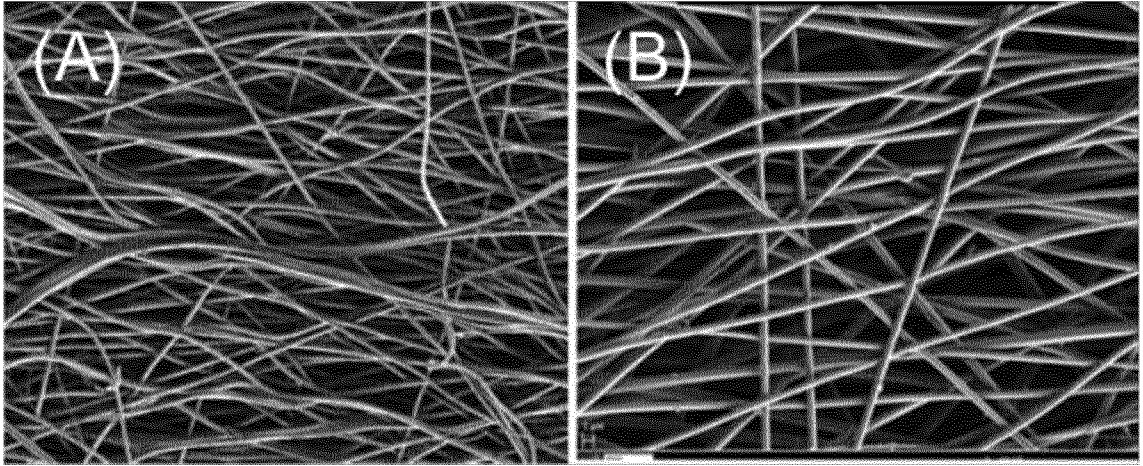


FIG. 10.

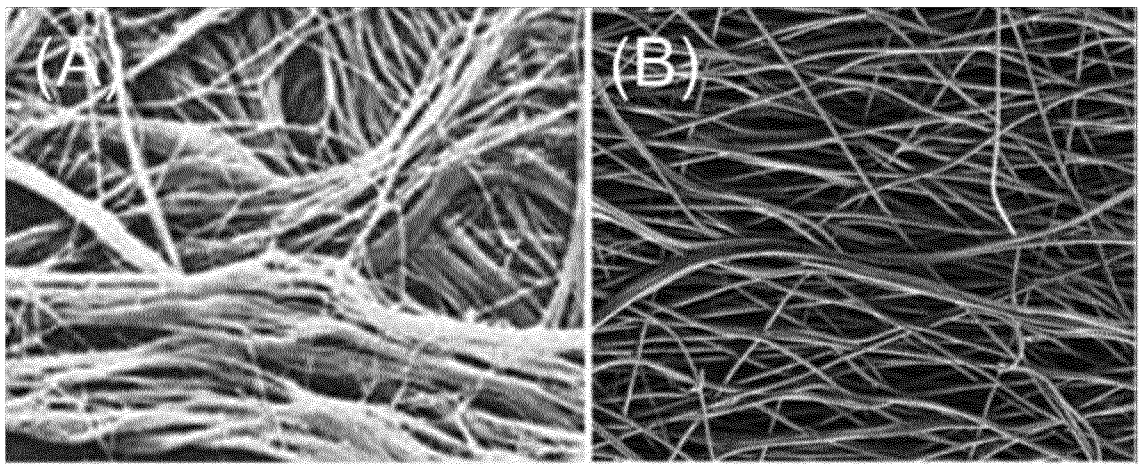


FIG. 11.

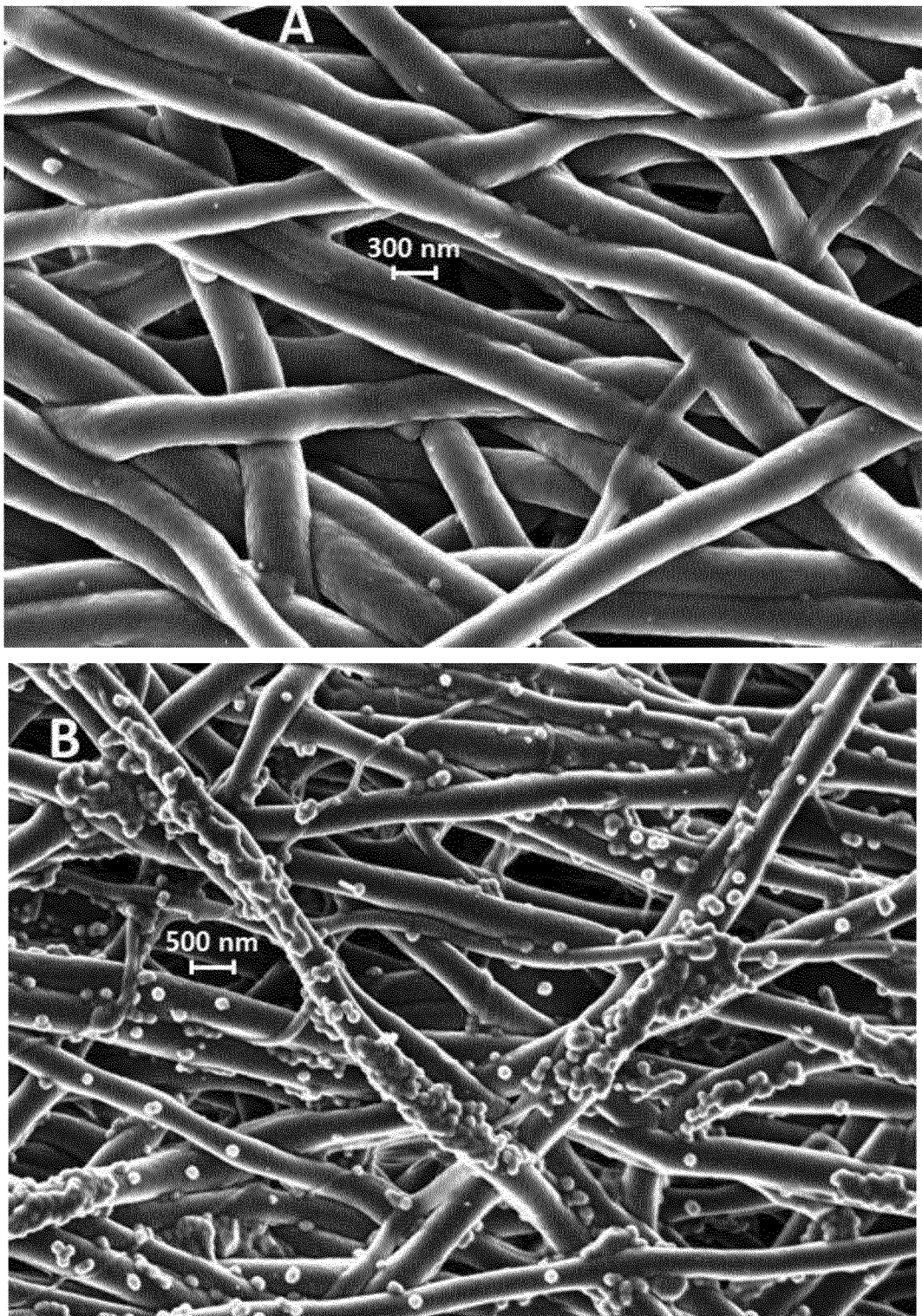


FIG. 11. Cont.

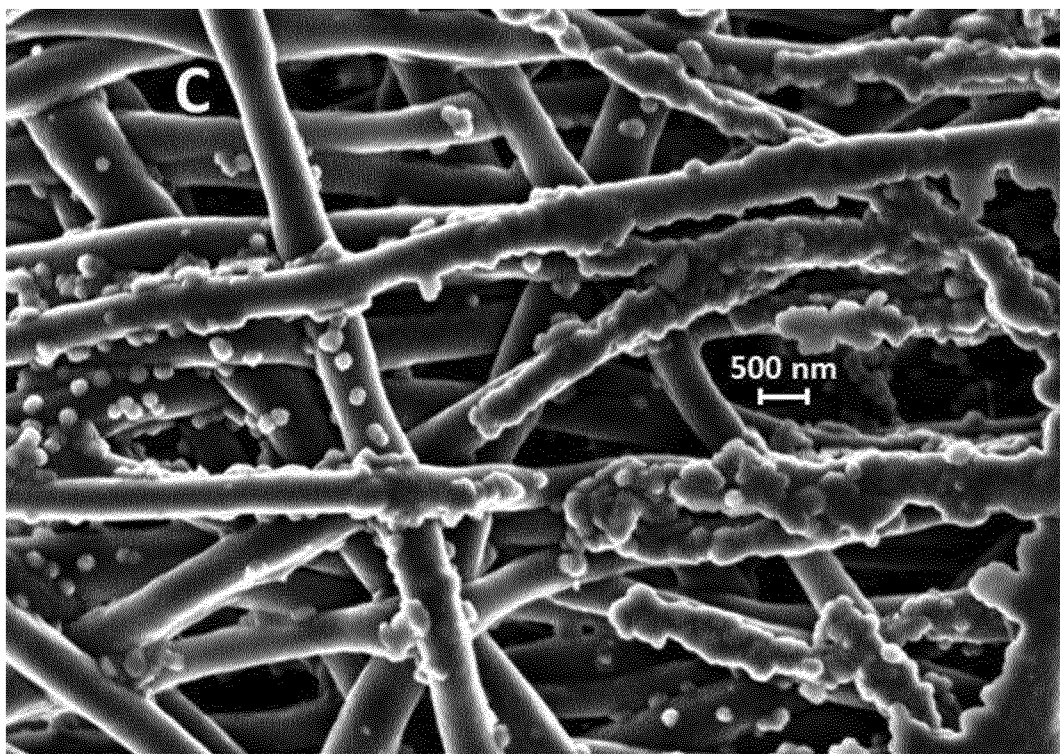


FIG. 12

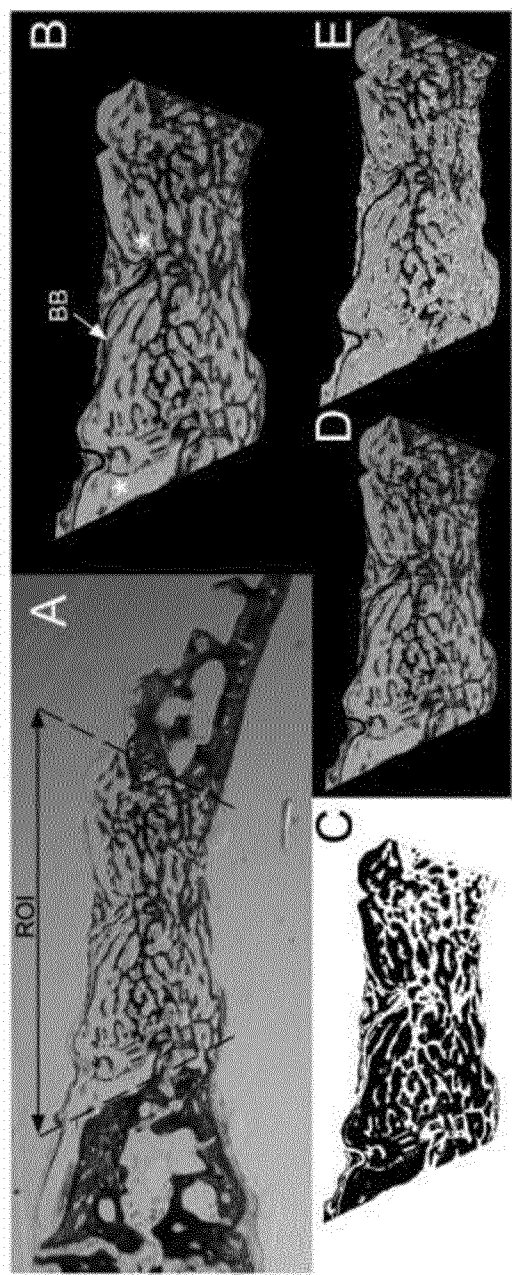


FIG. 13

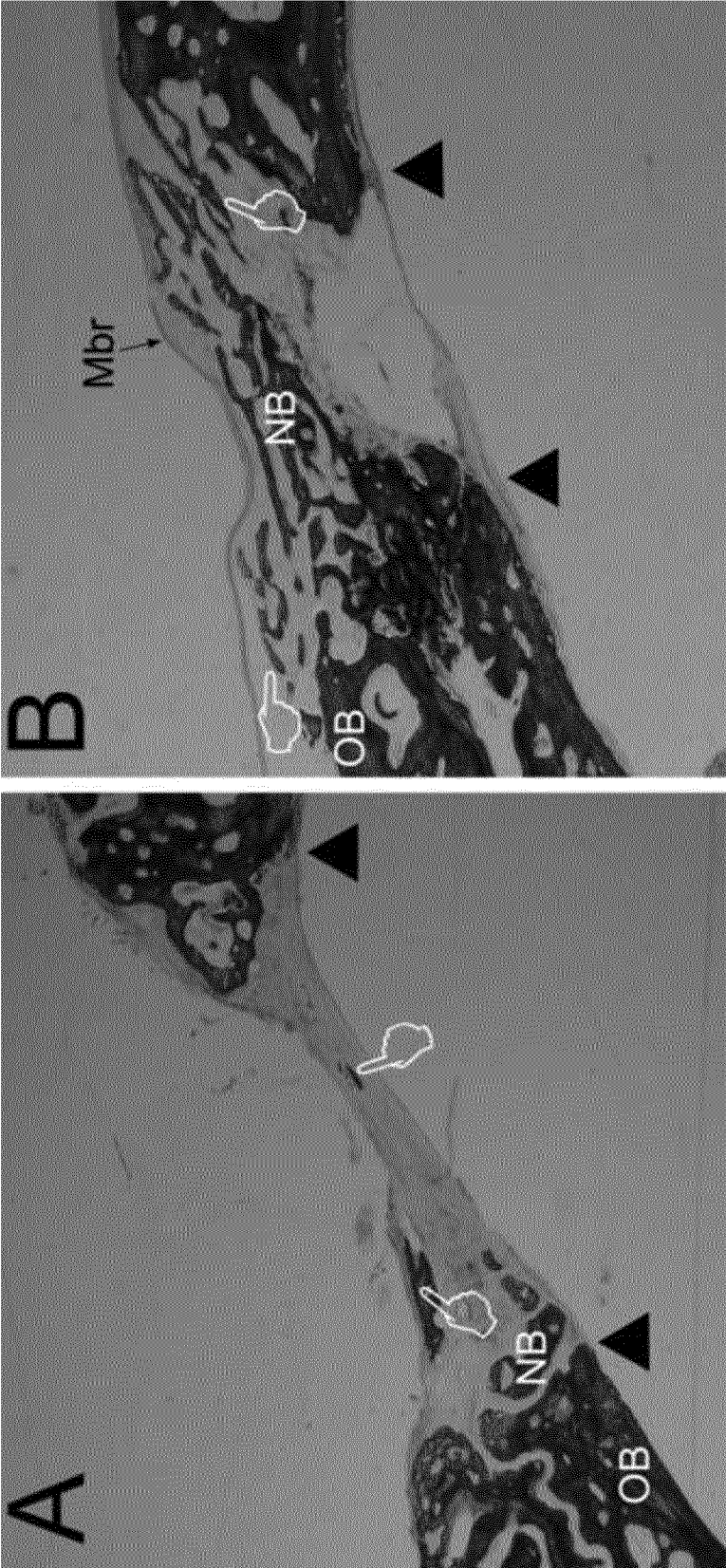


FIG. 14

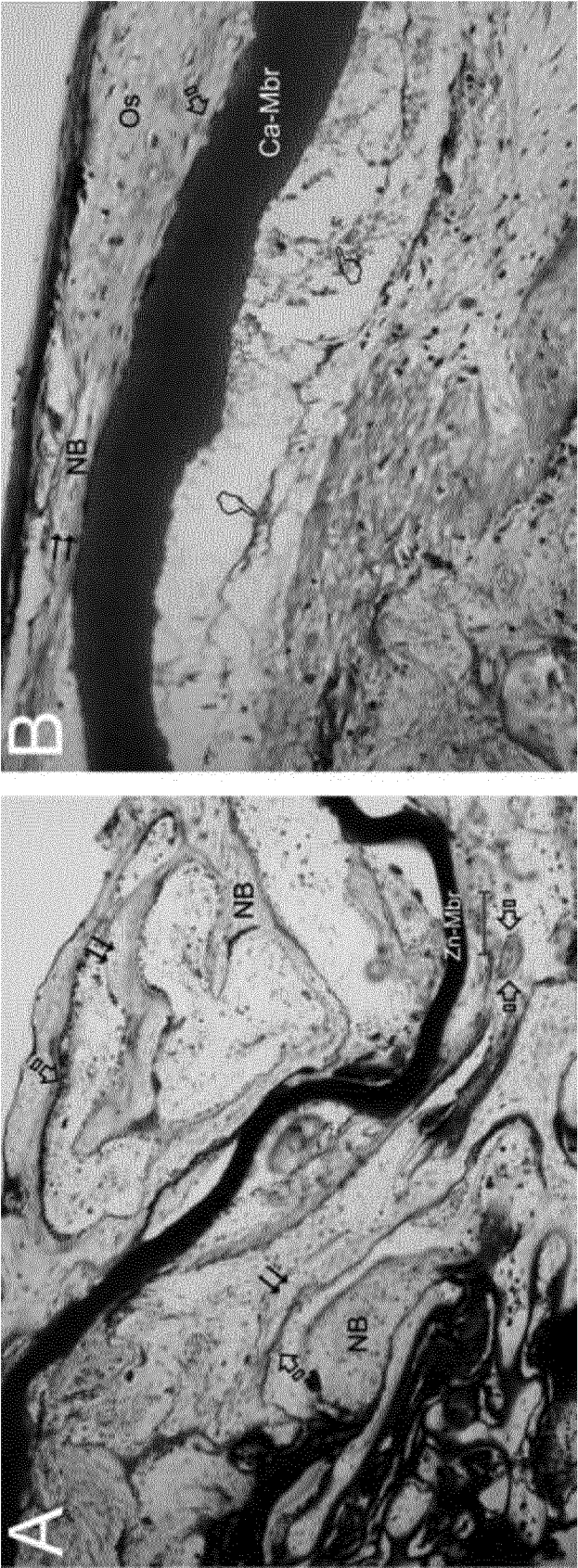


FIG. 15

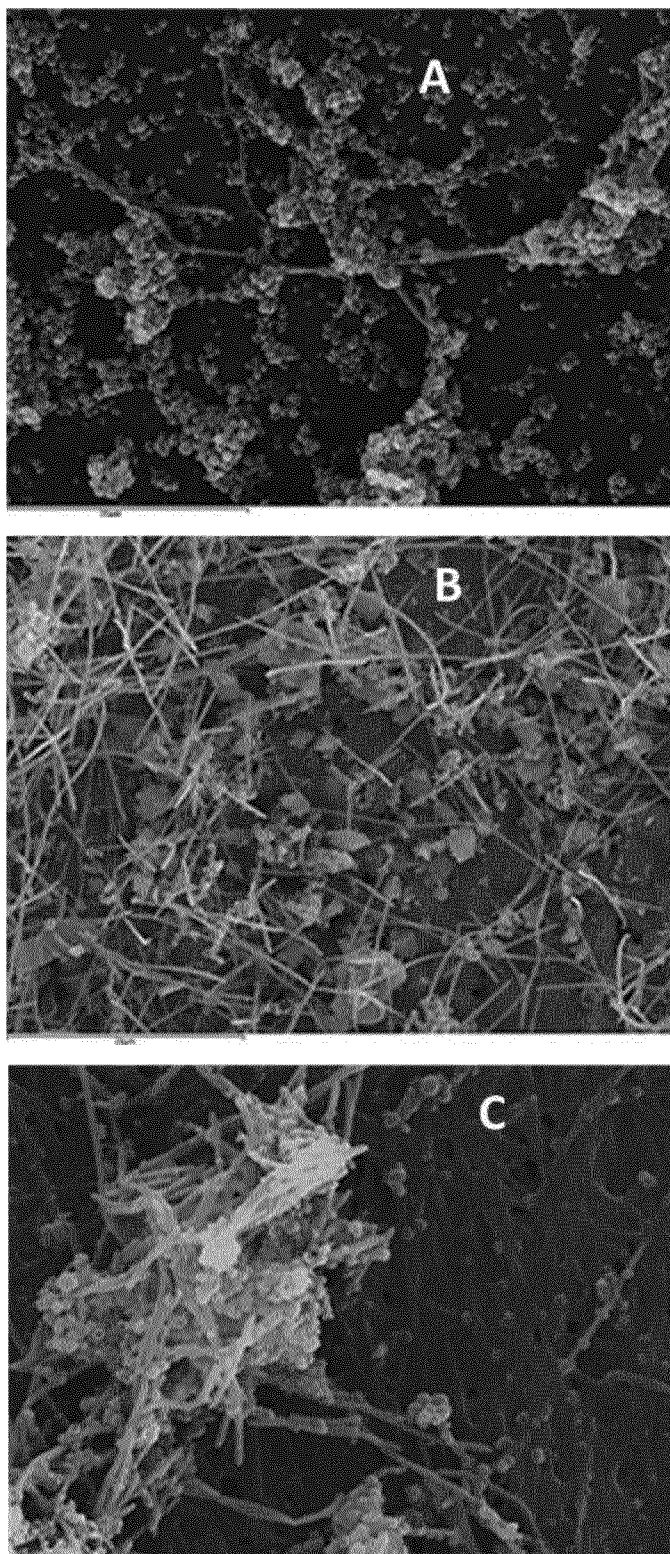


FIG. 15. Cont

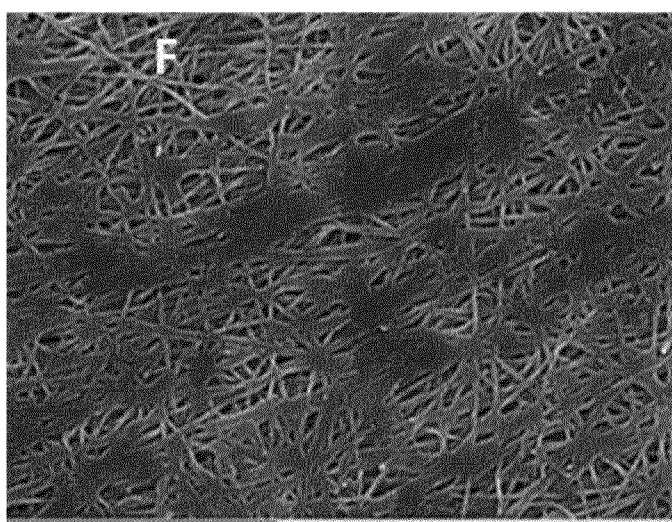
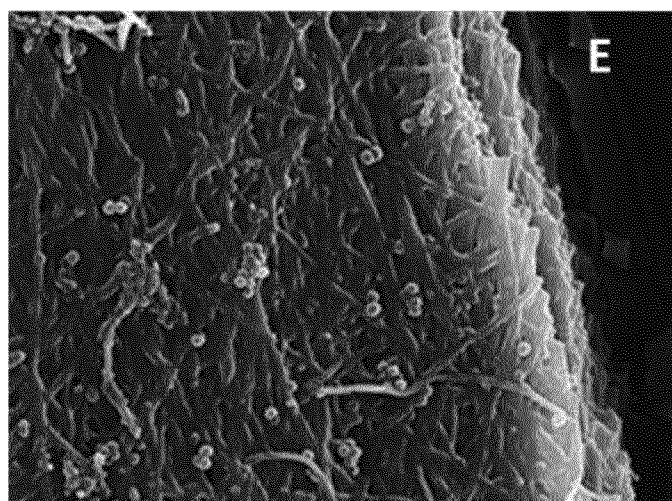
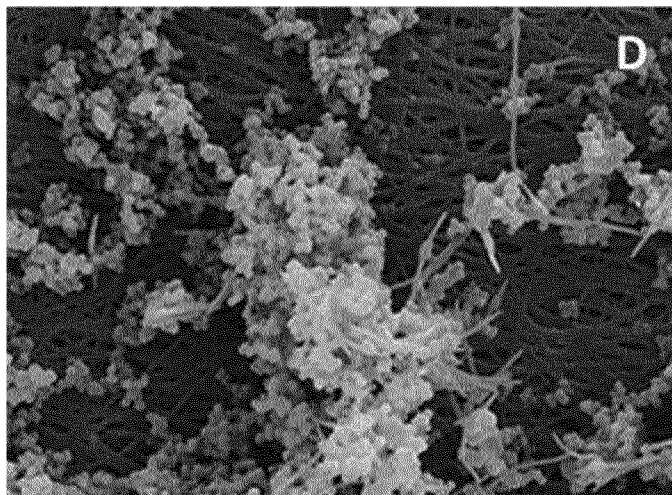


FIG. 16

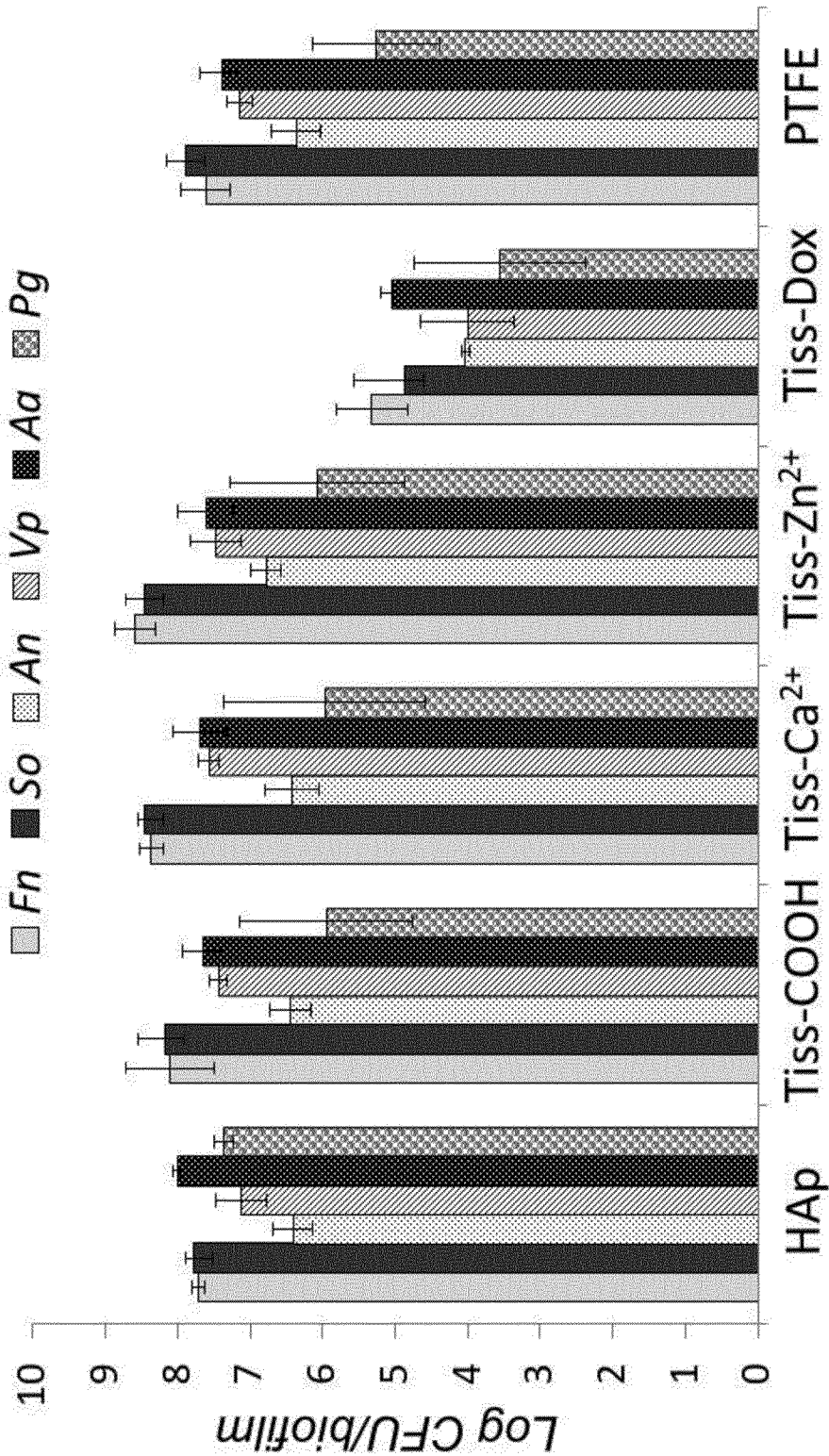


FIG. 17

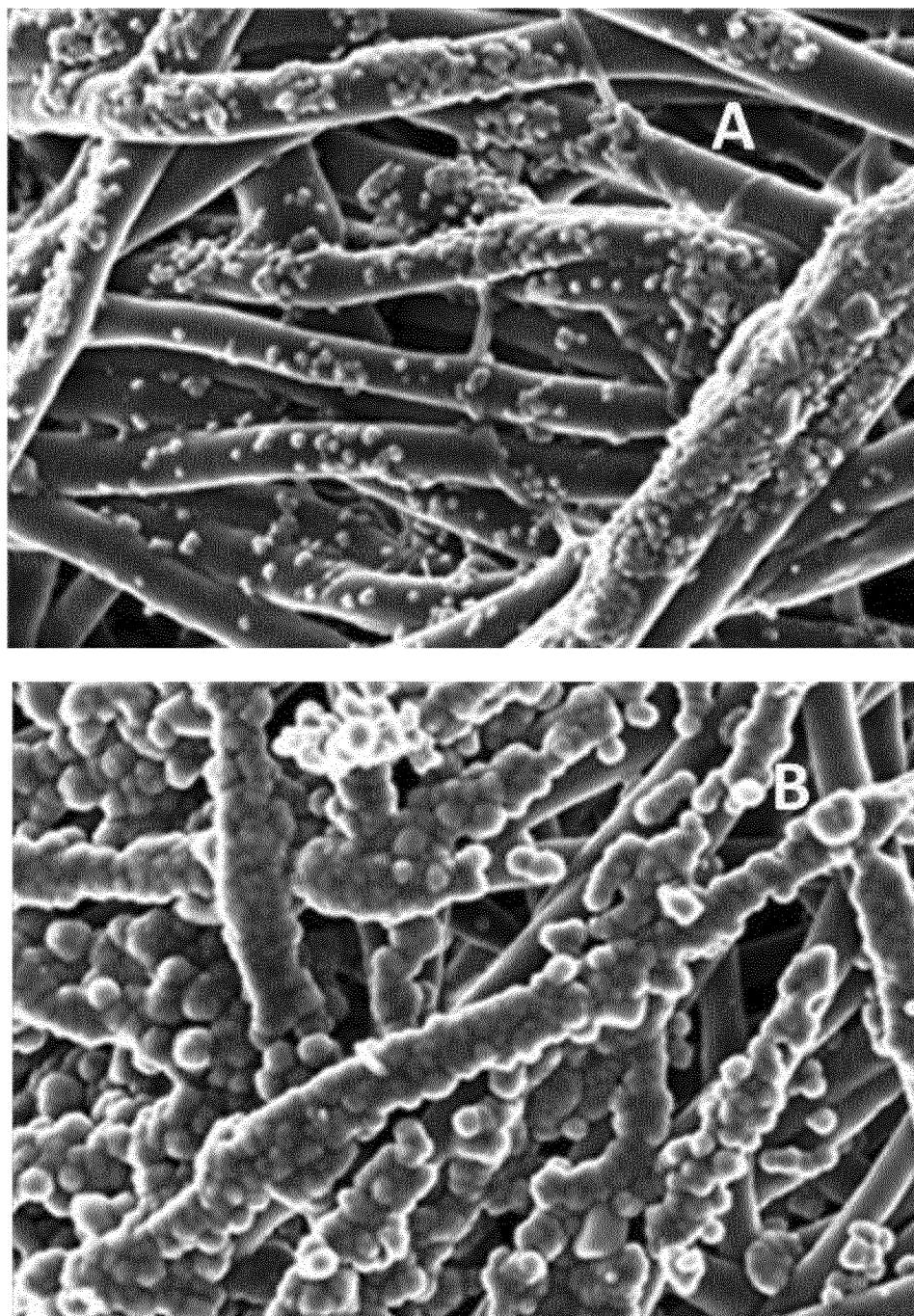
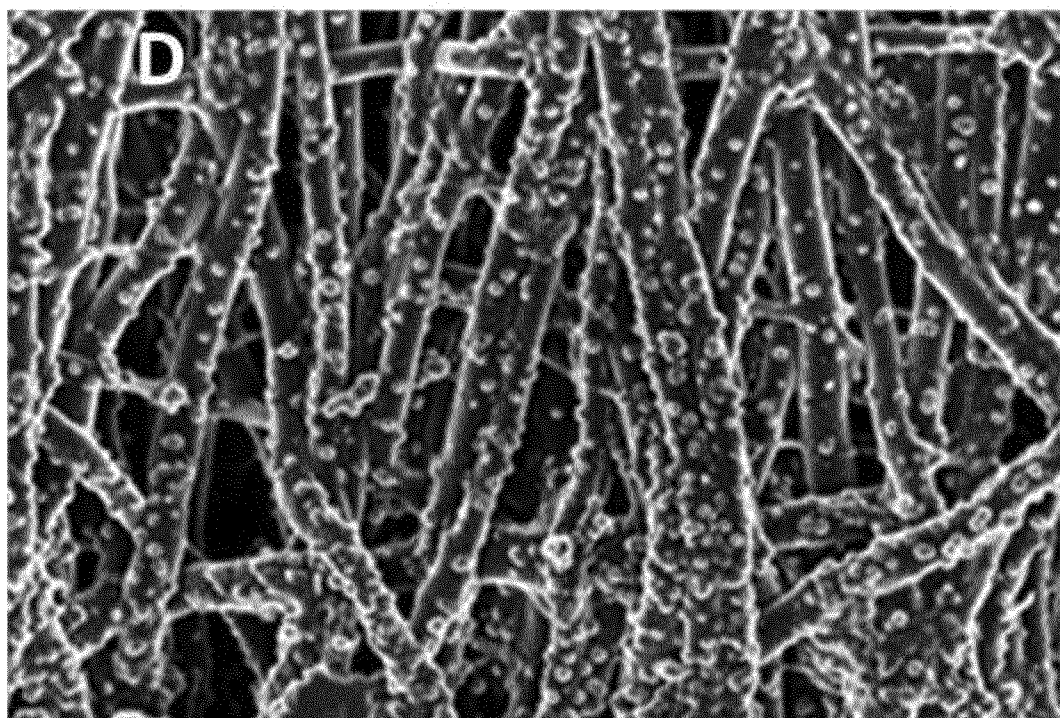
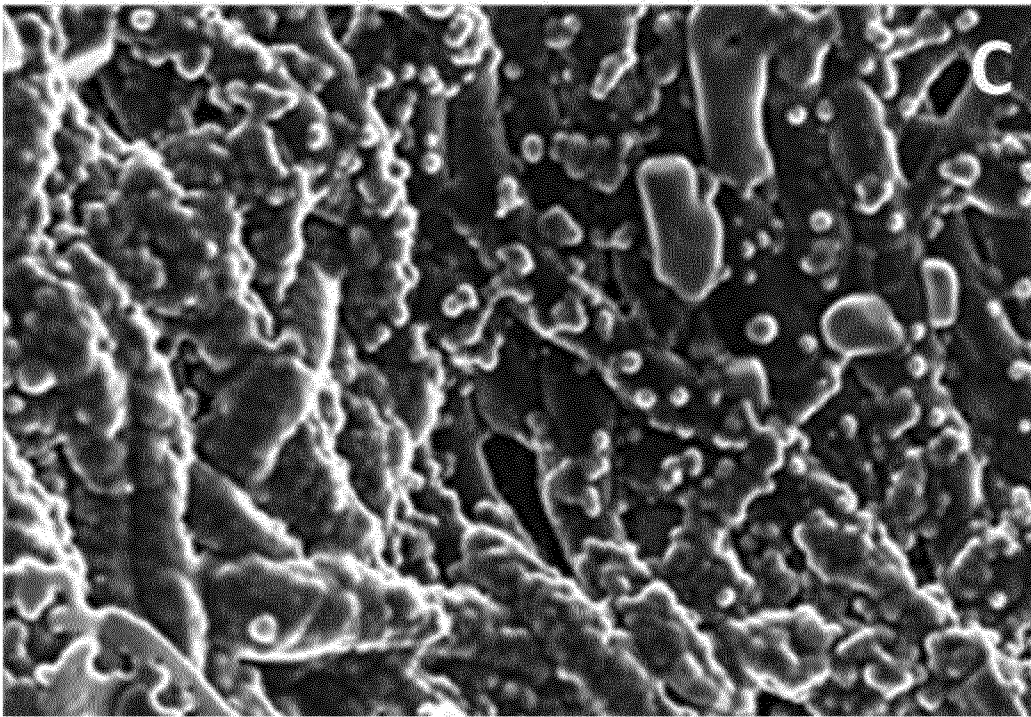


FIG. 17 Cont.



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2020/061184

A. CLASSIFICATION OF SUBJECT MATTER

INV. D04H1/435 A61K9/00 A61L27/16 A61L27/26 A61L27/50
D04H1/4382 D04H1/724

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)

D04H A61L A61K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2015/100120 A1 (TEPHA INC [US]) 2 July 2015 (2015-07-02) claims 1-28 -----	1-26
A	EP 0 569 797 A2 (DIGESTIVE CARE INC [US]) 18 November 1993 (1993-11-18) claims 1-32 -----	1-26
A	WO 2016/049682 A1 (UNIV GRIFFITH [AU]) 7 April 2016 (2016-04-07) claims 1-73 -----	1-26
A	KR 2015 0122444 A (KUMOH NAT INST TECH ACAD COOP [KR]) 2 November 2015 (2015-11-02) abstract; claims 1-13 ----- -/-	1-26

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

☒ See patent family annex.

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"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

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"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

24 July 2020

Date of mailing of the international search report

04/08/2020

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Fax: (+31-70) 340-3016

Authorized officer

Beins, Ulrika

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2020/061184

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CN 107 930 703 A (TONGXIANG JIACHE TECH CO LTD) 20 April 2018 (2018-04-20) abstract; claims 1-8 -----	1-26

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2020/061184

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		EP 3086820 A1	02-11-2016
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CN 107930703 A	20-04-2018	NONE	