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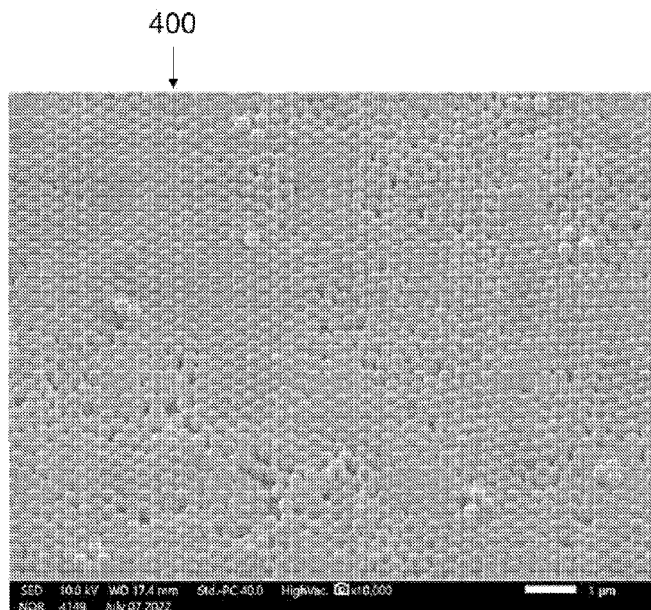


Figure 4

(57) Abstract: A composition for tissue regeneration in a subject, comprising a xenogeneic decellularized extracellular matrix (dECM) component containing a biological material, and a polymeric component, wherein the composition is produced by a method comprising: (i) isolating tissue from a donor animal to obtain the biological material; (ii) decellularizing the biological material to obtain the dECM component; and (iii) incorporating the dECM component with the polymeric component to produce the composition having enhanced antibacterial properties; and A method of producing a decellularized extracellular membrane (dECM) composition comprising a dECM component and a polymeric component for tissue regeneration in a subject, comprising the steps of: (i) obtaining tissue of interest from a donor animal; (ii) decellularizing the tissue of interest and removing genetic material from the tissue of interest to obtain the dECM component; and (iii) incorporating the dECM component with the polymeric component to produce the composition having enhanced

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antibacterial properties.

A COMPOSITION FOR TISSUE REGENERATION AND A METHOD OF PRODUCING THE SAME

CROSS-REFERENCE TO RELATED APPLICATION

The present application claims priority to United States Provisional Patent Application No. US63/497,006, the entire content of which is incorporated herein by reference.

TECHNICAL FIELD

This invention relates to a composition for application in tissue regeneration and wound healing, and a method of producing the same. In particular, the present disclosure is directed to a composition for tissue regeneration comprising (1) a xenogeneic decellularized extracellular matrix (dECM) component, and (2) a polymeric component, and wherein the composition has enhanced antibacterial properties.

BACKGROUND

dECM tissue regeneration harnesses decellularized extracellular matrix (dECM) scaffolds to facilitate tissue repair and regeneration. By removing cellular components while retaining the native extracellular matrix structure, dECM scaffolds or platforms serve as templates for cells to adhere, proliferate, and differentiate. These scaffolds have potential application in various fields, including tissue engineering and wound healing, offering advantages such as biocompatibility, bioactivity, and versatility.

A composition for application in tissue regeneration and wound healing that boosts the healing of tissue while reducing the challenges associated with post-operative infections, and a method of producing the same is desired.

SUMMARY OF THE INVENTION

Tissue engineering and regeneration aims to develop substitutes, constructs or platforms for damaged tissue requiring repair through the use of biomaterial scaffolds that mimic the natural extracellular matrix (ECM) environment. Decellularized extracellular matrix (dECM) scaffolds advantageously provide an effective biomimetic platform for regenerative medicine.

According to a first aspect of the invention, there is provided a composition for tissue generation in a subject, comprising a xenogeneic decellularized extracellular matrix (dECM) component containing a biological material, and a polymeric component, wherein the composition is produced by a method comprising:

- (i) isolating tissue from a donor animal to obtain the biological material;
- (ii) decellularizing the biological material to obtain the dECM component; and
- (iii) incorporating the dECM component with the polymeric component to produce the composition having enhanced antibacterial properties.

For example, the tissue is selected from one or more of foetal membrane or foetal bone from the donor animal. In a further example, the donor animal is foetal or neonatal goat. For example, the foetal or neonatal goat has a gestational age of about 12 weeks to about 18 weeks.

The composition of the claimed invention beneficially incorporates the biomimetic properties of the dECM component with a polymeric component that boosts its antibacterial properties thus accelerating the repair of injured or defective bone or wounded skin when used. The composition thus exhibits enhanced biocompatibility, customisability and versatility.

In an example embodiment, the composition includes a xenogeneic decellularized extracellular matrix (dECM) component containing a biological material. The biological material is obtained from tissue from a donor animal. For example, the tissue is selected from one or more of foetal membrane tissue and/or foetal bone tissue from the donor animal. The foetal membrane tissue includes, for example, one or more of amnion and/or chorion membranes. The foetal bone tissue includes, for example, one or more of cortical bone and/or cancellous bone. The use of xenogeneic dECM also provides the advantages of increased availability and is more widely accepted in view of religious concerns, etc. In an example embodiment, the dECM component is in the form of a powder, solution, hydrogel, scaffold, membrane, film, coating, or a combination thereof. The dECM component has a surface morphology that is porous, smooth, nanopatterned, or a combination thereof.

The dECM component is incorporated with a polymeric component. For example, the polymeric component is a film, scaffold, hydrogel, or a combination thereof. In a further example, the polymeric component has a surface morphology that is porous, smooth, nanopatterned, or a combination thereof. The nanopatterned morphology of the surface of the polymeric component and/or the dECM component beneficially provides the composition with enhanced antibacterial properties. In a further embodiment, the dECM component and/or the polymeric component comprises closely packed arrays of nanocones.

In an example embodiment, the polymeric component is derived from one or more natural polymers. These natural polymers include, but are not limited to, silk fibroin, chitosan, or a combination thereof. In an example embodiment, the silk fibroin is a stretchable film or a stiff film.

In another example embodiment, the polymeric component is pure chitosan or functionalised chitosan. For example, where the polymeric component is functionalised chitosan, the functionalised chitosan is created by mixing chitosan with N-(β -maleimidopropyl) succinimide ester for about 6 hours at room temperature.

In a preferred embodiment, the composition is a hydrogel, scaffold, membrane, or a coating.

In an example embodiment, the composition is applied to skin or bone tissue where tissue repair is required in a mammalian subject. For example, the skin in need of repair is wounded, such as a chronic wound or an infected skin wound, or burned, or a combination thereof. Additionally, the composition promotes and/or accelerates the repair of injured or defective bone tissue or wounded skin with effective antibacterial properties.

According to a second aspect of the invention, there is provided a method of producing a decellularized extracellular membrane (dECM) composition comprising a dECM component and a polymeric component for tissue regeneration in a subject, comprising the steps of:

- (i) obtaining tissue of interest from a donor animal;
- (ii) decellularizing the tissue of interest and removing genetic material from the tissue of interest to obtain the dECM component; and

(iii) incorporating the dECM component with the polymeric component to produce the composition having enhanced antibacterial properties. In a preferred embodiment, decellularizing the tissue of interest comprises treating the tissue of interest with chemical agents, including detergents, enzymes, chelating agents, or a combination thereof. For example, the detergent is Triton-X; the enzyme is DNase; the chelating agent is Ethylenediaminetetraacetic acid (EDTA). The dECM component is generated in a manner that prioritises maintaining architecture and bioactivity and the methods include the use of detergents, enzymes, chelating agents, mechanical approaches, and combinations thereof. Complete decellularization is further achieved by removing genetic material with nucleases to prevent host immune reactions. In an example embodiment, the dECM component is in a non-fragmented, fragmented, ground, or solubilized formats. In a further example embodiment, the dECM composition has a complex 3D structure, for example 3D scaffolds, spheroids, fibres or sheets.

In an embodiment, decellularizing the tissue of interest includes the following steps:

- (i) washing the tissue in a hypotonic buffer, optionally supplemented with an antibiotic, a chelating agent, and a protease inhibitor cocktail;
- (ii) washing the tissue in an isotonic buffer, optionally containing a detergent, a chelating agent, a protease inhibitor cocktail, and an antibiotic;
- (iii) washing the tissue with the isotonic buffer;
- (iv) washing the tissue in the hypotonic buffer; and
- (v) washing the tissue with the isotonic buffer and removing genetic material from the tissue of interest, resulting in the dECM component.

In an embodiment, steps (i) to (v) include shaking the tissue of interest at about 100 rpm to about 500 rpm. Additionally, decellularizing the tissue of interest includes an optional step of physically disrupting the tissue of interest by freeze-thawing, grinding, homogenization, osmotic shock, or a combination thereof.

In another embodiment, step (ii) includes a step of reducing a size of the tissue of interest, for example by grinding. Mechanically disrupting the tissue by freeze-thawing, grinding, or homogenization breaks down cell membranes and releases cellular components.

In a further embodiment, the polymeric component is derived from one or more natural polymers. For example, the polymeric component is silk fibroin, chitosan, or a combination thereof. Where the polymeric component is silk fibroin this is in a stretchable film or a stiff film form.

In an example embodiment, the polymeric component is pure chitosan or a functionalised chitosan. For example, the functionalised chitosan is created by mixing chitosan with N-(β -maleimidopropoxy) succinimide ester for about 6 hours at room temperature.

In an example embodiment, the polymeric component is a film, scaffold, hydrogel, or a combination thereof. Further, the surface morphology of the polymeric component and/or the dECM component is porous, smooth, nanopatterned, or a combination thereof. Preferably, a nanopatterned surface morphology of the dECM component and/or the polymeric component provides the dECM composition with enhanced antibacterial properties.

In a further embodiment, the dECM component is in the form of a powder, hydrogel, scaffold, membrane, film, coating, or a combination thereof.

In a preferred embodiment, the dECM composition is a scaffold, membrane, or a coating.

In an example embodiment, the donor animal is a foetal or neonatal goat and the tissue of interest is selected from one or more of foetal membrane or foetal bone from the donor animal. Preferably, the donor animal is a foetal or neonatal goat with a gestational age of about 12 weeks to about 18 weeks.

In one embodiment, the tissue of interest is selected from one or more of foetal membrane tissue and/or bone tissue from the donor animal. For example the foetal membrane tissue includes one or more of amnion and/or chorion membranes. In another example, the foetal or neonatal bone tissue includes one or more of cortical bone and/or cancellous bone.

In a preferred embodiment, the dECM composition is applied to skin or bone tissue of the subject where tissue repair is required, including but not limited to, for example repair of tissue which is vulnerable to infection. According to a further aspect of the invention, there is provided a method of repairing skin or bone tissue in a subject, the method comprising applying the dECM composition of claim 21 to the skin or bone tissue of the subject where tissue repair is required.

The claimed composition and method of producing the same exhibits enhanced biocompatibility, modularity, and versatility for tissue engineering applications.

BRIEF DESCRIPTION OF THE DRAWINGS

Embodiments of the present invention will now be described, by way of example, with reference to the accompanying drawings in which:

Figures 1A-1C are images showing foetal membranes (ACM) derived from goat, the epithelial layer along with the blood residues were removed by a cell scraper before applying the washing steps.

Figures 2A -2C are images showing freeze-dried dECM of goat ACM, Figure 2A shows the freeze-dried dECM before grinding and Figure 2B shows the freeze-fried dECM after grinding. Figure 2C shows pepsin-digested dECM component. The prepared solution was freeze-dried again to achieve dECM powder denoted as "dACM".

Figures 3A and 3B are images illustrating the gelation behaviour of the composite made of β MPs-modified chitosan hydrogel denoted as "BMPS" and dACM particles named "BMPS-dACM". The hydrogel solution is liquid and injectable at 25° (Figure 3A) while it can be crosslinked and turns into a stable hydrogel at 37 °C after incubation at -20 °C for 24 hours (Figure 3B).

Figure 4 is a scanning electron microscope (SEM) image of the coated polystyrene nanospheres on a plasticized silk substrate.

Figures 5A is a photograph of a nanopatterned, stretchable silk mesh and Figures 5B and 5C are SEM images showing the arrays of nanocones made of stretchable silk developed on the silk substrates.

Figure 6 is a photograph of a composite wound dressing scaffold ("ChitoSilkBioPatch") made by combining stretchable nanopatterned silk mesh and BMPS-dACM hydrogel.

Figure 7 is a graph showing the DNA contents of the non-decellularized tissue as control and the dECM component with the method of Example 1.

Figures 8A and 8B are H & E staining images of goat foetal membranes (ACM), Figure 8A is non-decellularized and Figure 8B is decellularized by the method of Example 1. The nuclei (black dots, indicated by arrows) are obvious in the control ACM while they can't be distinguished in the dECM component.

Figures 9A and 9B show trichrome staining of the foetal membranes (ACM), Figure 9A is non-decellularized as control and Figure 9B is the dECM component by the decellularization method of Example 1. The nuclei (black dots, indicated by arrows) are visible in the control ACM but are not visible in the dECM component.

Figure 10 is a graph showing the collagen content of the foetal goat membrane (ACM) samples before (control) and after the decellularization using the decellularization method of Example 1.

Figures 11A and 11B are graphs showing the compressive elastic modulus of stretchable silk substrates made with various ratios of CaCl₂ to formic acid kept in extreme dry conditions (desiccated)(Figure 11A) and normal atmospheric pressure condition (Figure 11B).

Figure 12 is a graph showing the compressive elastic modulus of the stretchable silk substrates made with a CaCl₂ to formic acid ratio of 3% kept in extreme dry conditions (desiccated), normal atmospheric pressure (non-desiccated), and wet-state conditions. The compressive elastic modulus of the substrate with nanocones on the surface in a wet-state condition is also shown.

Figure 13 shows photographs of agar plates onto which *S. aureus* bacterial cells were recultivated following: no treatment (control), treatment with pure chitosan hydrogel ("CS"), treatment with BMPS-chitosan hydrogel ("BMPS"), and treatment with BMPS-chitosan incorporated with dACM particles ("BMPS-dACM") for 1, 3, 6 and 24 hours.

Figure 14 is a graph showing anti-bacterial rates of the BMPS and BMPS-dACM relative to the CS hydrogel against *S. aureus* at different time points.

Figure 15 shows photographs of agar plates onto which *P. aeruginosa* bacterial cells were recultivated following: no treatment (control), treatment with CS, treatment with BMPS, and treatment with BMPS-dACM, for 1, 3, 6 and 24 hours.

Figure 16 shows photographs of agar plates onto which *S. aureus* bacterial cells were recultivated following: no treatment (control), treatment with silk, and treatment with nanopatterned silk, for 1, 3, 6 and 24 hours.

Figure 17 shows photographs of agar plates onto which *P. aeruginosa* bacterial cells were recultivated following: no treatment (control), treatment with silk, and treatment with nanopatterned silk, for 1, 3, 6 and 24 hours.

Figures 18A-18D are SEM images of *S. aureus* bacterium cultured on silk (Figures 18A and 18B) and nanopatterned silk (Figures 18C and 18D) for 6 hours confirming the anti-bacterial effect of silk nanocones against *S. aureus* compared to the said silk substrate without nanofeatures.

Figures 19A-19D are SEM images of *S. aureus* bacterium cultured on silk (Figures 19A and 19B) and nanopatterned silk (Figures 19C and 19D) for 24 hours confirming the anti-bacterial effect of silk nanocones against *S. aureus* compared to the silk substrate without nanofeatures for up to 24 hours.

Figures 20A-20D are SEM images of *P. aeruginosa* bacterium cultured on silk (Figures 20A and 20B) and nanopatterned silk (Figures 20C and 20D) for 3 hours confirming the anti-bacterial effect of silk nanocones against *P. aeruginosa* compared to the silk film without nanopatterns.

Figures 21A-21D are SEM images of *P. aeruginosa* bacterium cultured on silk (Figures 21A and 21B) and nanopatterned silk (Figures 21C and 21D) for 24 hours, confirming the significant anti-bacterial effect of silk nanocones against *P. aeruginosa* compared to the silk substrate without nanopatterns for up to 24 hours.

Figures 22A and B shows dynamic change of diabetic wound areas after full-thickness skin defect surgery. In Figure 22A, there are illustrated macroscopic observation of diabetic wounds in each group at day 0, 1, 4, 7, 10 and 14, and quantitative evaluation of wound closure rate on days 10 and 14 after different wound dressings treatment. In Figure 22B, there is shown H&E staining results of wound beds in each group at day 7 and 14. * p<0.05, ** p<0.01 vs. NC group. ^ p<0.05, ^^ p<0.01 vs. CS group. # p<0.05, ## p<0.01 vs. BMPS group. & p<0.05, && p<0.01 vs. BMPS-dACM group. n=6/group/time point.

Figures 23A to C shows Collagen deposition of diabetic wound areas after full-thickness skin defect surgery. In Figure 23A, there is shown Sirius Red staining results of wound beds in each group at day 7 and 14. In Figure 23B, there is shown Quantitative evaluation of collagen I positive area, collagen III positive area and collagen I/ collagen III ratio based on Sirius Red staining at day 7. C. Quantitative evaluation of collagen I positive area, collagen III positive area and collagen I/ collagen III ratio based on Sirius Red staining at day 14. * p<0.05, ** p<0.01 vs. NC group. ^ p<0.05, ^^ p<0.01 vs. CS group. # p<0.05, ## p<0.01 vs. BMPS group. & p<0.05, && p<0.01 vs. BMPS-dACM group. n=6/group/time point.

Figures 24A to 24D shows macrophages subset detection of diabetic wound areas on day 7 after full-thickness skin defect surgery. In Figure 24A, there is shown double labelling IF staining of F4/80 and iNOS of wound beds in each group on day 7. In Figure 24B, there is shown double labeling IF staining of F4/80 and CD206 of wound beds in each group on day 7. In Figure 24C, there is shown a semi-quantitative analysis of F4/80 and iNOS double-positive cells. In Figure 24D, there is shown a semi-quantitative analysis of F4/80 and CD206 double-positive cells. * p<0.05, ** p<0.01 vs. NC group. ^ p<0.05, ^^ p<0.01 vs. CS group. # p<0.05, ## p<0.01 vs. BMPS group. & p<0.05, && p<0.01 vs. BMPS-dACM group. n=6/group/time point.

Figures 25A and 25B shows Neovascular formation of diabetic wound areas on day 14 after full-thickness skin defect surgery. In Figure 25A, there is shown double labeling IF staining of CD31 and α -SMA of wound beds in each group on day 14. In Figure 25B, there is shown a semi-quantitative analysis of blood vessels, which are labelled as CD31 and α -SMA double positive. * $p < 0.05$, ** $p < 0.01$ vs. NC group. ^ $p < 0.05$, ^^ $p < 0.01$ vs. CS group. # $p < 0.05$, ## $p < 0.01$ vs. BMPS group. & $p < 0.05$, && $p < 0.01$ vs. BMPS-dACM group. n=6/group/time point.

Figures 26A-26D shows images of harvested bone from goat foetus after removing the muscle, fat, and skin (Figure 26A), which were chopped into small pieces using surgical tools (Figure 26B) and then ground into small particles for decellularization (Figure 26C). Figure 26D is an image of the foetal goat bone dECM component after being freeze-dried.

Figure 27 is a graph showing the DNA content of foetal goat bone, non-decellularized as control or decellularized with methods of Example 2 including foetal bone dECM (dECM) and demineralized foetal bone dECM (demineralized dECM).

Figures 28A-28C show H & E staining images of the foetal goat bone samples. Figure 28A is a non-decellularized control, Figure 28B is decellularized ECM, and Figure 28C is decellularized and demineralized bone ECM produced by the decellularization and/or demineralization methods of Example 2. The nuclei (black dots, indicated by arrows) are obvious in the control tissue but can't be distinguished in the dECM and demineralized dECM.

Figures 29A-29C show trichrome staining of the foetal goat bone membranes. Figure 29A is a non-decellularized control, Figure 29B is decellularized ECM, and Figure 29C is decellularized and demineralized ECM produced by the decellularization and/or demineralization methods of Example 2. The nuclei (black dots, indicated by arrows) are visible in the control but not visible in the dECM, although the collagen fibers have been well-preserved during the decellularization and demineralization process.

Figure 30 shows SEM images of non-reinforced foetal goat bone dECM-silk membranes prepared at different ratios of silk to demineralized bone dECM digest.

Figures 31A and 31B show SEM images of the nanopatterned non-reinforced dECM-silk (1:1) illustrating the fibrillar morphology of the membrane with the arrays of hexagonally closely packed nanocones on the surface.

Figures 32A-32D illustrate the morphology of the 2% dECM-silk hybrid composite (Figures 32A and 32B) and its nanopatterned counterpart (Figures 32C and 32D). The yellow arrows indicate the dispersion of foetal bone dECM particles that are $\leq 200 \mu\text{m}$ in the substrate.

Figure 33 shows photographs of agar plates onto which *S. aureus* bacterial cells were recultivated after treatment with a negative control and with nanopatterned 2% dECM-silk hybrid composite for 3 hours.

Figure 34 shows photographs of agar plates onto which *S. aureus* bacterial cells were recultivated after treatment with a negative control, O_2 plasma-etched non-reinforced dECM-silk (1:1), nanopatterned non-reinforced dECM-silk (1:1), O_2 plasma-etched 2% dECM-silk hybrid composite, nanopatterned 2% dECM-silk hybrid composite, and ciprofloxacin as a positive control at a concentration of (20 mg/mL), for 1, 3, 6 and 24 hours.

Figures 35A-35D are SEM images of *S. aureus* bacterium cultured on O_2 plasma-etched non-reinforced dECM-silk (1:1) (Figures 35A and 35B) and nanopatterned non-reinforced dECM-silk (1:1) (Figures 35C and 35D) for 3 hours confirming the anti-bacterial properties of both samples as the bacterial cell walls have been damaged when cultured on the surfaces. However, the synergistic effects of both the nanocones which interact with cell walls and the dECM AMPs of the foetal demineralized bone used in the substrate of nanopatterned non-reinforced dECM-silk (1:1) caused considerably less bacterial attachment to the surface.

Figures 36A-36D are SEM images of *S. aureus* bacterium cultured on O_2 plasma-etched 2% dECM-silk hybrid composite (Figures 36A and 36B) and nanopatterned 2% dECM-silk hybrid composite (Figures 36C and 36D) for 3 hours confirming the anti-bacterial properties of both samples as the bacterial cell walls have been damaged when cultured on the surfaces. However, the synergistic effects of both the nanocones which interact with cell walls and the dECM AMPs of the foetal bone (mineral and demineralized) used in the substrate of the nanopatterned 2% dECM-silk hybrid composite caused much less bacterial attachment to the surface with more significant bacterial membrane damage.

Figures 37A-37D are SEM images of *S. aureus* bacterium cultured on O_2 plasma-etched non-reinforced dECM-silk (1:1) (Figures 37A and 37B) and nanopatterned non-reinforced dECM-silk (1:1) (Figures 37C and 37D) for 24 hours confirming the anti-bacterial properties of both samples as the bacterial cell walls were damaged when cultured on the surfaces. However, the synergistic effects of both the nanocones which interact with cell walls and the dECM AMPs of the foetal demineralized bone used in the substrate of the nanopatterned non-reinforced dECM-silk (1:1) caused less bacterial attachment to the surface.

Figures 38A-38D are SEM images of *S. aureus* bacterium cultured on O_2 plasma-etched 2% dECM-silk hybrid composite (Figures 38A and 38B) and nanopatterned 2% dECM-silk hybrid composite (Figures 38C and 38D) for 24 hours confirming the anti-bacterial properties of both samples as the bacterial cell walls were damaged when cultured on the said surfaces. However, the synergistic effects of both the nanocones and the dECM AMPs of the foetal bone (mineral and demineralized) used in the substrate of the nanopatterned 2% dECM-silk hybrid composite caused less bacterial attachment to the surface with more significant bacterial membrane damage. Almost none of the bacteria on the surface are alive with only bacteria debris visible.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

Definitions

As used herein, the term "subject" refers to an animal, needing or desiring delivery of the benefits provided by a therapeutic compound. As used herein, the term "animal" may be, for example, humans, pigs, horses, goats, cats, mice, rats, dogs, apes, fish, chimpanzees, orangutans, guinea pigs, hamsters, cows, sheep, birds, chickens, as well as any other vertebrate or invertebrate. These benefits can include, but are not limited to, the treatment of a health condition, disease or disorder; prevention of a health condition, disease or disorder; immune health; enhancement of the function of an organ, tissue, or system in the body. The preferred subject in the context of this invention is a human. The subject can be of any age or stage of development.

As used herein, the term “treatment” refers to eradicating, reducing, ameliorating, or reversing a sign or symptom of a health condition, disease or disorder to any extent, and includes, but does not require, a complete cure of the condition, disease, or disorder. Treating can be curing, improving, or partially ameliorating a disorder. “Treatment” can also include improving or enhancing a condition or characteristic, for example, bringing the function of a particular system in the body to a heightened state of health or homeostasis.

As used herein, the term “cellular components” refers to cell membranes, cytoplasm, dsDNA, and organelles (e.g., nucleus, mitochondria, endoplasmic reticulum, Golgi apparatus, lysosome) that make up a cell.

As used herein, the term “decellularized ECM (dECM)” refers to eliminating cellular constituents and the related debris upon digestion including DNA, RNA, and cell nuclei, for example, from the ECM. The terms “acellular” ECM or tissue for instance foetal membranes (amniotic and/or chorionic membrane) and/or bone, refers to “dECM”, of the correlated tissue and they might use interchangeably. As used herein, the term “decellularization,” refers to the substantial (i.e., at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or greater) removal of cellular components by the use of chemical means. Decellularization can be measured by quantification of DNA content per unit of weight of dried extracellular cellular matrix (ECM).

As used herein, the term “composition” and/or “dECM composition” refers to any biomaterial, scaffold, construct, film, membrane, hydrogel, powder, particle, implant, drug delivery system, platform, composite, or any medical device that is composed of at least one type of dECM.

As used herein the terms “scaffold”, “bio-scaffold”, “implant”, “bio-implant”, “construct”, “bio-construct”, “composite”, “bio-composite”, “medical device” and “biomedical device” might be used interchangeably.

As used herein, a “scaffold” refers to a temporary or permanent structure used as a support system to aid in the growth and regeneration of tissue in damaged or diseased areas. A “bio-scaffold”, on the other hand, is a scaffold made from biocompatible materials, which can support the growth of living tissue and eventually integrate with the body's natural tissues.

As used herein the terms “foetal membranes” refers to the combination of “amniotic and chorionic membranes” or “amnio-chorionic membrane (ACM)” which might be used interchangeably. As used herein, “foetal membranes” refer to the membranes that surround and protect the developing foetus during pregnancy. There are two main foetal membranes: the amniotic membrane and the chorionic membrane. The amniotic membrane is the innermost membrane that surrounds the developing foetus and is filled with amniotic fluid, which provides cushioning and protection for the developing foetus. The chorionic membrane is the outermost membrane that is in contact with the uterine wall and helps to form the placenta, which provides nutrients and oxygen to the developing foetus.

As used herein, the terms “amnion” refers to the “amniotic membrane” which might be used interchangeably. As used herein, the “amnion” refers to a thin, tough, and transparent membrane that forms the innermost layer of the foetal membranes that surround and protect the developing embryo or foetus during pregnancy. It is filled with amniotic fluid, which serves several important functions, such as cushioning the foetus, maintaining a constant temperature, and providing a medium for foetal movement and growth.

As used herein the terms “chorion” refers to the “chorionic membrane” which might be used interchangeably. As used herein, the “chorion” refers to one of the two foetal membranes that surround and protect the developing embryo or foetus during pregnancy. It forms the outermost layer of the foetal membranes and is located next to the uterus. The chorion plays a crucial role in the development of the placenta, which is the organ that provides the foetus with oxygen and nutrients and removes waste products. The chorionic villi, which are finger-like projections that extend from the chorion into the uterine lining, allow for the exchange of nutrients, oxygen, and waste products between the mother and the foetus.

As used herein the terms “substrate”, “film” and “membrane” might be used interchangeably.

As used herein the terms “nanopatterns”, “nanofeatures” and “nano topographies” might be used interchangeably. As used herein, a “nanopattern” refers to a pattern on the surface of a material/structure that has at least one dimension in the nanometer range (typically below 100 nm). Nanopatterns can be created by various methods such as using nano-sized materials/particles, chemical patterning, self-assembly, photolithography, nanoimprint lithography, colloidal lithography, soft lithography, 3D bioprinting etc. These nano-scaled patterns can have uniform distribution over surface of the material with a wide range of shapes including dots, pits, columns, pillars, lines, gratings, or complex geometries. As used herein, the term “nanofeature” generally refers to a feature or a geometric structure having at least one nanoscale dimension (between 1 nm to 1000 nm) which can have different shapes (grooves, grides, pits, pillars, cones, etc.). As used herein, the term “nano topography” refers to the array of surface features of a material or substrate with at least one dimension in submicron size or nanoscale (1 to 1000 nm). In the context of tissue engineering and regenerative medicine, nano topographies can be used to regulate cell fate and tissue growth.

As used herein the terms “hydrogel” and “gel” might be used interchangeably, which refers to 3D polymeric structure that is insoluble in liquid (like water) while being capable of absorbing large amount of liquid to form a steady and often soft and flexible structure.

Decellularized extracellular matrix (dECM) provide unique advantages that make it well suited for application in tissue engineering and regenerative medicine. Some of these advantages include that the dECM retains the same biochemical composition and biomechanical properties of the donor tissue from which it is derived, advantageously providing high biocompatibility and bioactivity. dECM signalling mimics the natural microenvironment of the donor tissue and thus growth factors, cytokines, and cell adhesion peptides in the dECM beneficially promotes and enables cell adhesion, proliferation, differentiation, and tissue remodelling.

dECM can be used in tissue engineering and regenerative medicine as a biomimetic scaffold composition, biomaterial, or bio-construct that supports and promotes the regeneration of functional tissues and organs. A dECM-based composition beneficially facilitates cell migration, proliferation, and tissue remodelling. It advantageously promotes angiogenesis, the formation of new blood vessels, and recruits endogenous stem cells to the site of injury, enhancing the regenerative capacity of damaged tissues.

Further, by varying factors such as selecting tissues from different donors or sources, altering decellularization methods, and incorporating additional components like polymers, dECM-based compositions, scaffolds, gels, or films can be tailored to match the mechanical, structural, and biochemical requirements of specific tissues or organs.

With reference to the Figures, an embodiment of the present invention is illustrated. This embodiment is arranged to provide a composition for tissue regeneration in a subject, comprising a xenogenic decellularized extracellular matrix (dECM) component containing a biological material, and a polymeric component, wherein the composition is produced by a method comprising:

- (i) obtaining tissue from a donor animal for the biological material;
- (ii) decellularizing the biological material to obtain the dECM component; and
- (iii) incorporating the dECM component with the polymeric component to produce the composition having enhanced antibacterial properties. In certain embodiments, the composition is a scaffold, membrane, or a coating.

In certain embodiments, the donor animal is a foetal or neonatal goat. For example, the foetal or neonatal goat has a gestational age of about 12 weeks to about 18 weeks. In certain embodiments, the tissue is selected from one or more foetal membrane or foetal bone from the donor animal, for example foetal membrane tissue and/or foetal bone tissue such as one or more of amnion and/or chorion membranes and one or more of cortical bone and/or cancellous bone, respectively.

Figures 1A to 1C show foetal membranes (ACM) derived from goat where the epithelial layer along with the blood residues were removed by a cell scraper before applying the washing steps.

Figure 26A-26D show images of harvested bone from goat foetus after removing the muscle, fat, and skin (Figure 26A, 2600), which were chopped into small pieces using surgical tools (Figure 26B, 2610) and then ground into small particles for decellularization (Figure 26C, 2620). Figure 26D 2630 shows foetal goat bone dECM component 2630 after being freeze-dried.

In an example embodiment, the dECM component 200 is in the form of a powder, solution, hydrogel, scaffold, membrane, film, coating, or a combination thereof. In certain embodiments, the dECM can be cooled, using, for example, liquid nitrogen to reduce the temperature of the tissues before applying the grinding process to avoid destruction of the dECM. After or during the grinding process, the dECM can be washed by rinsing the tissue with water or a water-based solution and/or a detergent. Before being placed in the grinder container, the dECM can be immersed in liquid nitrogen to be snap-frozen and freeze dried. The grinding of the tissues can occur for at least about 5 s, about 10 s, about 15 s, about 20 s, about 25 s, about 30 s, about 35 s, about 40 s, about 45 s, about 60 s, about 90 s, about 120 s, wherein after a time interval, such as, for example, about 5 s, 10 s, or 15 s, the samples that are being ground can be immersed in liquid nitrogen again to avoid destruction of dECM proteins by the heat generated by the grinder. In certain embodiments, the dECM resulting from the ACM tissue can be digested in a pepsin solution (such, as for example, about 1 mg to about 2.5 mg or about 2 mg in each mL of 0.01 M hydrochloric acid, HCL) at room temperature (e.g., about 18°C to about 25°C) under stirring for about 24 h to about 72 h or about 48 h. The resulted digest can be neutralized using a base and an isotonic buffer (e.g., 10X phosphate buffered saline (PBS), saline, 3-(N-morpholino) propanesulfonic acid (MOPS), HEPES, or Hank's Balanced Salt Solution). In certain embodiments, the neutralized tissue can be snap frozen and freeze-dried. In certain embodiments, the tissue can be then be sterilized, by, for example, ultraviolet light.

Figures 2A – 2C are images showing freeze-dried dECM component 200 before grinding (Figure 2A), after grinding (Figure 2B), and pepsin-digested dECM component 200.

The composition has enhanced antibacterial properties that are intrinsic and arise from the dECM component 200, the polymeric component 300,500, or a combination thereof. For example, the enhanced antibacterial properties arise from the composition and/or surface morphologies of the dECM component 200 and/or the polymeric component 300,500, and advantageously provide a composition that does not require the administration or inclusion of antibiotic agents in the composition, thus minimising the risk of employing high dosages of chemical and antibiotics which might cause or contribute to toxicity and antimicrobial resistance.

In certain embodiments, a surface morphology of the dECM component 200 and/or the polymeric component 300,500 is porous, smooth, nanopatterned, or a combination thereof. In a preferred embodiment, the nanopatterned surface morphology of the dECM component 200 and/or the polymeric component 300,500 provides the composition with enhanced antibacterial properties. In certain embodiments, the dECM component 200 and/or the polymeric component 300,500 comprises closely packed arrays of nanocones. The closely packed nanocones 520 are illustrated in Figures 5B 510 and 5C 520 showing the arrays made of stretchable silk developed on the polymeric component 500.

In another embodiment the polymeric component 300, 500 is derived from one or more natural polymers. For example, the polymeric component 300, 500 is silk fibroin, chitosan, or a combination thereof. Where the polymeric component 300, 500 is silk fibroin, the silk fibroin is a stretchable film or a stiff film. Where the polymeric component 300, 500 is chitosan, the chitosan is pure chitosan or a functionalised chitosan. For example, the functionalised chitosan created by mixing chitosan with N-(β-maleimidopropoxy) succinimide ester for about 6 hours at room temperature.

Figures 3A and 3B illustrates chitosan as the polymeric component 300. The images illustrate the gelation behaviour of the BMPS-modified chitosan hydrogel incorporated with dACM particles ("BMPS-dACM" hydrogel 300). The hydrogel solution is liquid and injectable at 25° (Figure 3A) while it can be crosslinked and turns into a stable hydrogel after incubation at -20 °C for 24 hours (Figure 3B).

In a further embodiment, the polymeric component 300, 500 is a film, scaffold, hydrogel, or a combination thereof.

In a preferred embodiment, the composition is applied to skin or bone tissue of a subject where tissue repair is required. For example, where the skin in need of repair is wounded or burned, wherein the wound is a chronic wound, an infected skin wound, or a combination thereof. The composition of the claimed invention advantageously promotes and/or accelerates the repair of injured or defective bone tissue or wounded skin with effective antibacterial properties.

A further embodiment of the present invention relates to a method of producing a decellularized extracellular membrane (dECM) composition comprising a dECM component and a polymeric component for tissue regeneration in a subject, comprising the steps of:

- (i) obtaining tissue of interest from a donor animal;
- (ii) decellularizing the tissue of interest and removing genetic material from the tissue of interest to obtain the dECM component; and
- (iii) incorporating the dECM component with the polymeric component to produce the composition having enhanced antibacterial properties.

In preferred embodiments, the tissue of interest is selected from one or more of foetal membrane or foetal bone. The foetal membrane tissue includes one or more of amnion and/or chorion membranes. In certain embodiments, the bone is cancellous or cortical bone or a combination thereof which are harvested from femora, tibiae, or a combination thereof.

In an example embodiment, the donor animal is a foetal or neonatal goat. Preferably, the donor animal is a foetal or neonatal goat has a gestational age of about 12 weeks to about 18 weeks

When isolating the tissue of interest, in certain embodiments, frozen tissues can be thawed at 4°C overnight. In certain embodiments, the blood residues and the epithelial layer can be separated by, for example, a cell scraper, and disposed. In certain embodiments, the skin, muscle, fat and any other tissues, except bone, can be separated by surgical tools and disposed.

In certain embodiments, decellularizing the tissue of interest comprises treating the tissue of interest with chemical agents, including detergents, enzymes, chelating agents, or a combination thereof. For example, the detergent is triton-x, enzyme is DNase, and the chelating agent is EDTA. For example, when decellularizing the tissue of interest tissue is incubated in a buffer, such as, for example, hypotonic tris buffer (e.g., 10 mM HEPES, pH 7.9, with 1.5 mM MgCl₂ and 10 mM KCl; 20 mM Tris-HCl, pH 7.4 with 10 mM NaCl and 3 mM MgCl; 10 mM HEPES, pH 7.5 with 10 mM MgCl₂ and 20 mM KCl; or 5 mM Tris-HCl, pH 7.4 with 3 mM EDTA), optionally supplemented with an antibiotic (e.g., 1% Pen-Strep), a chelating agent (e.g., ethylenediaminetetraacetic acid, (EDTA, 0.1% w/v) or 1,10-phenanthroline) and protease inhibitor cocktail (e.g., at a concentration of about 2.95 µl/mL). In certain embodiments, the incubated ACM tissue can then be washed in an isotonic buffer (e.g., tris-buffered saline (TBS, pH: about 7.6), phosphate buffered saline (PBS), or N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)), optionally, containing an ionic detergent (e.g., sodium dodecyl sulfate (SDS, 1% w/v), Triton X-100, Triton X-200, sulfobetaine-10 and -16, Tween 80, or 3-((3-cholami- dopropyl) dimethylammonio)-1-propanesulfonate (CHAPS)), a chelating agent (e.g., EDTA (0.1% w/v) or 1,10-phenanthroline), protease inhibitor cocktail (e.g., at a concentration of about 2.95 µl/mL) and an antibiotic (e.g., 1% Pen-Strep), for about 4 h to about 72 h or about 24 h using a shaking incubator at about 250 rpm and room temperature. In certain embodiments, the ACM tissues can be washed with an isotonic buffer (e.g., PBS, HEPES, or TBS) at a pH of about 7.6 at least 1, 2, 3, 4 or more times until the bubbles in the buffer were eliminated. In certain embodiments, the ACM tissue can be washed with a hypotonic lysis buffer, such as, for example, Tris-HCl (e.g., at a concentration of about 50 mM), about 10 mM magnesium chloride, and 50 µg/mL bovine serum albumin at a pH of about 7.5 to about 7.6, optionally supplemented with a DNase, such as, for example, DNase I at concentration of about 100 U/mL, an RNase, such as, for example, RNase A at a concentration of about 2 U/mL and an antibiotic (e.g., 1% Pen-Strep), for about 30 min to about 12 h or about 3 h at 250 rpm and 37°C. In certain embodiments, the hypotonic buffer can be 10 mM HEPES, pH 7.9, with 1.5 mM MgCl₂ and 10 mM KCl; 20 mM Tris-HCl, pH 7.4 with 10 mM NaCl and 3 mM MgCl; 10 mM HEPES, pH 7.5 with 10 mM MgCl₂ and 20 mM KCl; or 5 mM Tris-HCl, pH 7.4 with 3 mM EDTA. In certain embodiments, after removing the previous buffer, the tissues can be washed with an isotonic buffer (e.g., PBS, HEPES, or TBS) about 2 times to about 5 times or about three times. In certain embodiments, the TBS can be removed the ACM tissues can be resuspended in sterile milliQ H₂O, resulting in decellularized extracellular matrix (dECM).

In a further embodiment, decellularizing the tissue of interest includes the following steps:

- (i) washing the tissue in a hypotonic buffer, optionally supplemented with an antibiotic, a chelating agent, and a protease inhibitor cocktail;
- (ii) washing the tissue in an isotonic buffer, optionally containing a detergent, a chelating agent, a protease inhibitor cocktail, and an antibiotic;
- (iii) washing the tissue with the isotonic buffer;
- (iv) washing the tissue in the hypotonic buffer; and
- (v) washing the tissue with the isotonic buffer and removing genetic material from the tissue of interest, resulting in the dECM component.

For example, decellularizing the tissue of interest includes an optional step of physically disrupting the tissue of interest by freeze-thawing, grinding, homogenization, osmotic shock, or a combination thereof. This breaks down cell membranes in the tissue and releases cellular components.

In certain embodiments, the dECM resulting from the ACM tissue can be digested in a pepsin solution (such, as for example, about 1 mg to about 2.5 mg or about 2 mg in each mL of 0.01 M hydrochloric acid, HCl) at room temperature (e.g., about 18°C to about 25°C) under stirring for about 24 h to about 72 h or about 48 h. The resulted digest can be neutralized using a base and an isotonic buffer (e.g., 10X phosphate buffered saline (PBS), saline, 3-(N-morpholino) propanesulfonic acid (MOPS), HEPES, or Hank's Balanced Salt Solution). In certain embodiments, the neutralized tissue can be snap frozen and freeze-dried. In certain embodiments, the tissue can be then be sterilized, by, for example, ultraviolet light.

In certain embodiment, step (ii) includes a step of reducing a size of the tissue of interest, for example by grinding the tissue. In certain embodiments, during or immediately after the washing or freeze-drying, the dECM component resulting from the tissue can be reduced in size, by, for example, grinding. In certain embodiments, the dECM can be cooled, using, for example, liquid nitrogen to reduce the temperature of the tissues before applying the grinding process to avoid destruction of the dECM. After or during the grinding process, the dECM can be washed by rinsing the tissue with water or a water-based solution and/or a detergent. Before being placed in the grinder container, the dECM can be immersed in liquid nitrogen to be snap-frozen and freeze dried. The grinding of the tissues can occur for at least about 5 s, about 10 s, about 15 s, about 20 s, about 25 s, about 30 s, about 35 s, about 40 s, about 45 s, about 60 s, about 90 s, about 120 s, wherein after a time interval, such as, for example, about 5 s, 10 s, or 15 s, the samples that are being ground can be immersed in liquid nitrogen again to avoid destruction of dECM proteins by the heat generated by the grinder.

In an example embodiment, steps (i) to (v) include shaking the tissue of interest at about 100 rpm to about 250 rpm. In a further example embodiment, step (i) is performed for about 4 hours to about 72 hours at room temperature and step (v) is performed for about 30 minutes to about 12 hours at 37°C.

In certain embodiments, the polymeric component 300, 500 is derived from one or more natural polymers, including silk fibroin, chitosan, or a combination thereof. The silk fibroin is, for example, a stretchable film or a stiff film. The chitosan, for example, is pure chitosan or functionalised chitosan. In an example embodiment, the chitosan is functionalised chitosan created by mixing chitosan with N-(β-maleimidopropyl)oxy succinimide ester for about 6 hours at room temperature.

The polymeric component 300, 500 is a film, scaffold, hydrogel, or a combination thereof and the surface morphology of the polymeric component 300, 500 is porous, smooth, nanopatterned, or a combination thereof.

In certain embodiments, the dECM component 200, 2630 is in the form of a powder, solution, hydrogel, scaffold, membrane, film, coating, or a combination thereof. Further, surface morphology of the dECM component 200, 2630 is porous, smooth, nanopatterned, or a combination thereof.

The nanopatterned surface morphology of the dECM component and/or the polymeric component provides the dECM composition with enhanced antibacterial properties thus providing a dECM composition with antibacterial and anti-inflammatory properties and well as optimal mechanical properties. The dECM composition is a scaffold, membrane, or a coating that can be applied to skin or bone tissue of the subject where tissue repair is required.

In certain embodiments, the subject invention relates to the use of dECM for fabricating anti-bacterial bio-constructs. In one embodiment, the present invention provides the compositions, and methods of making the same, for wound healing purposes, such as, for example, in diabetic patients. In certain embodiments, the invention provides the compositions, and methods of making the same, for bone regeneration purposes.

In certain embodiments, the invention provides a dECM-based medical device with improved anti-bacterial and biological properties for tissue regeneration and wound healing applications comprising a dECM component and a polymeric component.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Any reference to prior art contained herein is not to be taken as an admission that the information is common general knowledge, unless otherwise indicated.

EXAMPLES MATERIALS AND METHODS

Foetal Membrane dECM

Foetal membrane is a thin bilayered structure including amniotic membrane and chorionic membrane which surrounds the developing foetus during pregnancy. The amnion and chorion are separable and offer different roles during pregnancy. While the amnion covers the embryo and holds the amniotic fluid, the chorion surrounds the amnion, embryo, and other membranes, acting as a protective barrier during foetal development.

The amniotic membrane is a jelly-like avascular matrix without nerves and lymph vessels closest to the foetus. It is composed of three layers including an epithelial layer, basement layer and avascular stromal layer which in turn comprises of a compact, a fibroblast and a spongy layer connected to the chorion. In most of the performed studies, the basement membrane of amnion has been used for the majority of tissue engineering applications. This membrane mainly consists of collagen, which provides structural integrity and mechanical strength to the tissue. Other than type III, IV, and V collagen, non-collagenous glycoproteins are also present in its structure, including fibronectin, laminin, and nidogen. An array of growth factors can be found in the amniotic membrane, such as keratinocyte growth factor (KGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), transforming growth factor (TGF)- α , - β 1, - β 2 and - β 3, KGF receptor (KGFR), hepatocyte growth factor (HGF), HGF receptor (HGFR), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF).

Chorion is the outer layer of the foetal membrane, which is also in contact with the mother's cells and consists of three layers of a) reticular layer made of collagens I, III, IV, V, and VI and proteoglycans, b) basement membrane comprising collagen IV, fibronectin, and laminin, and c) a trophoblasts layer. Both chorion and amnion contain variable amounts of cytokines and growth factors as shown by the proteomic evaluation on amnion and chorion. For example, in chorion factors such as adiponectin, angiopoietin-2, bFGF, Endocrine gland-derived vascular endothelial growth factor (EG-VEGF), HGF, insulin-like growth factor (IGF-1), (tissue inhibitor of metalloproteinase) TIMP-2, and TIMP-4, PDGF-AA and PDGF-BB have been detected. Therefore, foetal membrane and its derivatives, amnion, chorion have been used to develop biomaterials over 100 years for many tissue engineering and regenerative medicine purposes. Human amniotic membrane is very popular for ocular surface reconstruction or cornea replacement in ophthalmology, for the treatment of chronic wounds and burns, or as skin substitutes in dermatology. Also, human amnio-chorionic membrane (ACM) has shown promising outcomes in wound healing applications although its usage is less common than amniotic membrane.

Foetal membranes are beneficial for wound healing applications due to the existing proteins and growth factors in their ECMs. The ACM usually protects the foetus from pathogens which can cause dangerous pregnancy complications, including premature rupture of membranes and preterm delivery. One of the underlying mechanisms of such protection is their structural impermeability toward pathogens. Besides this, the existence of antimicrobial peptides (AMPs) which are expressed by the cells in the membranes directly affect the microorganisms available in amniotic fluid and ACM. AMPs are a group of small proteins that are secreted by some immune and epithelial cells and play important roles in the innate immune system. They provide anti-bacterial, anti-inflammatory, anti-fungal, anti-viral, anti-fibrotic, and analgesic effects along with regulating cell differentiation and angiogenesis, all of which are crucial in the wound healing process.

Amniotic membrane's growth factors such as TGF- β and EGF stimulate the migration of keratinocytes. The secreted factors stimulate both keratinocytes and fibroblasts during the proliferative phase of wound healing. They can also affect the migration of keratinocytes which leads to the wound re-epithelialization. Besides this, as it has both anti- and pro-angiogenic properties, it can regulate angiogenesis. Furthermore, during the remodeling phase it boosts wound contraction and scar development.

Despite the many advantages of human-derived amniotic membrane, its usage is limited due to a number of factors, including: (1) it is scarce, and its use is cost-prohibitive, (2) it has weak mechanical properties, (3) it can cause infectious disease, and (4) religious concerns can prevent widespread use. Hence, we address these limitations by choosing xenogeneic-derived foetal membrane dECMs rendering the possibility of mass production while delivering regenerative properties for wound healing purposes.

Decellularization of goat foetal membrane

Frozen foetal goat membranes (ACM) were thawed overnight at 4°C followed by removal of the blood residues and the epithelial layer gently using a cell scraper (Figures 1A-1C). The membranes were then chopped into small pieces and incubated in hypotonic tris buffer (10 mM, pH:8) containing ethylenediaminetetraacetic acid, (EDTA, 0.1% w/v) and protease inhibitor cocktail (2.95 µL/mL) and penicillin-streptomycin-neomycin antibiotic mixture (PSN, 1%) for 16 hours. Subsequently, the tissues were washed with tris-buffered saline (TBS, pH: 7.6) containing sodium dodecyl sulfate (SDS, 1% w/v), EDTA (0.1% w/v), protease inhibitor cocktail (2.95 µL/mL) and PSN (1%) for 24 hours using a shaking incubator at 250 rpm and room temperature. Next, the tissues were washed with TBS at pH 7.6 several times until the bubbles in the buffer were eliminated. The samples were then washed with a buffer made of 50 mM Tris-HCL, 10 mM magnesium chloride and 50 µg/mL bovine serum albumin at pH 7.5 supplemented with 100 U/mL DNase I, 2 U/mL RNase A and 1% PSN for 3 hours at 250 rpm and 37 °C. After removing the previous buffer, the tissues were washed with TBS three times and lastly TBS was exchanged with sterile MilliQ H₂O. The samples were snap frozen in liquid N₂ and freeze-dried (Figure 2A).

Fabrication of pepsin-digested foetal membrane dECM particles

The freeze-dried foetal membrane dECM was ground by a grinder (IKA A11 basic) to reduce the size of the dECM pieces into smaller particles (Figure 2B). The dECM pieces were incorporated in the grinder container while being immersed in liquid nitrogen for a while before grinding to avoid destruction of dECM proteins upon the heat generated by the grinding process. Then, 20 mg of the dried ACM dECM powder was digested in a pepsin solution (2 mg in 0.01 M hydrochloric acid, HCL) at room temperature under stirring for 48 h. The resultant digest (FIG. 2C) was then neutralized by 0.1 M sodium hydroxide (NaOH) and 10X phosphate buffered saline (PBS) on ice, snap frozen and freeze-dried. The achieved powder was UV-sterilized before usage for in vitro and animal studies.

Chemical functionalization of chitosan

In order to improve the anti-bacterial properties of the chitosan, the chitosan chains were chemically functionalized with maleimide groups using a maleimide-NHS ester cross-linking reagent named N-(β-maleimidopropoxy) succinimide ester (βMPS). βMPS is a water insoluble, heterobifunctional protein cross-linker presenting thiol- and amine-reactive groups linked by a 5.9 Å spacer arm. Through its NHS-ester, it can be conjugated with (or label) the primary amines of proteins, amine-modified oligonucleotides or other amine containing molecules, while through its maleimide group it reacts with a substance or biomolecule with thiol group and forms a covalent bond. Here, βMPS directly coupled to the chitosan amino groups by amide formation, rendering a controlled degree of chitosan chemical modification. In addition to the said mechanism, the remaining chitosan amino groups further react with the maleimido groups provided by βMPS through Michael addition which proceeds mildly without forming side products. As a result of the said chemical reactions, the βMPS-chitosan can serve as a scaffold. Not only is βMPS-chitosan able to self-crosslink but also it has improved anti-bacterial properties compared to the non-functionalized chitosan. To chemically functionalize the chitosan with βMPS, oligochitosan with molecular weight of MW=5~10 kDa, Cat. No. P312015 was dissolved in heated PBS (at pH 7.4) with concentration of 4 %w/v. Later, the solution was centrifuged to remove the undissolved powder at 7800 rpm for 20 min, and the supernatant was filtered using syringe-driven filters of 0.45 and 0.22 µm, sequentially. After adjusting the pH to 7.0 by NaOH solution (1 M), 4 mL of βMPS solution (308.59 mM) in dimethyl formamide was added dropwise to the chitosan solution gradually while the solution was mixed thoroughly using a magnetic stirrer at 1000 rpm. After 6 hours of stirring at room temperature, the achieved solution was filtered using syringe-driven filters of 0.45 and 0.22 µm, sequentially and dialyzed against ultrapure water for 3 days by a dialysis bag (MWCO: 3.5 kDa). Subsequently, the solution was concentrated by means of centrifugal filters (3 kDa MWCO) to tune the final chitosan concentration to 3 %w/v. Next, the solution impurities were removed by 0.22-µm filter, and the obtained βMPS-chitosan solution was flash frozen rapidly by liquid nitrogen and kept at -80 °C.

Fabrication of chitosan-based hydrogels

To fabricate non-functionalised chitosan hydrogels, β-glycerophosphate (β-GP) was solubilized in ultrapure water and exploited as a physical crosslinking agent for pure oligochitosan solution in PBS with chitosan and β-GP concentrations of 3 %w/v and 14.2 wt.%, respectively. Subsequently, the mixture was incubated at 37 °C for several minutes to develop a stable hydrogel. This sample was named as "CS". Also, βMPS-chitosan (chitosan concentration of (3 %w/v)) was incubated at -20°C for 24 hours to form a stable hydrogel named as "BMPS". Both CS and BMPS were used to investigate the effects of chitosan chemical functionalization and dACM utilization on the chitosan-based gels anti-bacterial properties. To develop, the composite hydrogel, βMPS-chitosan (chitosan concentration of (3 w/v%)) was mixed with pepsin-digested ACM dECM powder at a concentration of 20mg per mL. Upon mixing by using a vortex mixer, the dECM particles were almost dissolved in the βMPS-chitosan solution and evenly distributed within the chitosan polymer network in several seconds and a homogenous solution was achieved. Later, the mixture was incubated at -20°C for 24 hours to develop a stable hydrogel (Figures 3A and 3B). This sample was denoted as "BMPS-dACM".

Fabrication of stretchable silk fibroin films

To fabricate stretchable silk fibroin substrates, first silk fibroin was produced. A 5g quantity of the silkworm (*Bombyx mori*) cocoons were cut and boiled in water (2L) supplemented with sodium carbonate (Na₂CO₃, 0.02M) for 30 minutes to degum the silk and remove its sericin. The achieved silk was then washed with ultrapure water three times and dried at room temperature. Later, constant amount of dried silk (1.5 g) was dissolved in 10g of formic acid containing calcium chloride (CaCl₂) at different ratios of CaCl₂ to formic acid (2, 2.5, 3 and 5%). After dissolving the silk fibres, the trapped bubbles were removed by ultrasonication followed by casting in the desired mould. The cast moulds were placed under a fume hood for several days by which the formic acid in the cast solutions was allowed to evaporate completely. Next, the silk substrates were detached from their moulds and water annealed overnight to crosslink them appropriately. Subsequently, the substrates were washed with ultrapure water to remove any formic acid residue on the surface for 30 minutes.

Developing nanopatterns on the plasticized silk substrates

Colloidal lithography was performed on the water annealed silk membrane with the best mechanical properties wherein the ratio of CaCl₂ to formic acid was 3% . For this purpose, 2.5 w/v% solution of polystyrene nanospheres (from Tianjin Baseline Chrom Tech Research Center (Product No. 6-1-0030), China) with size of 300 nm were used. Few drops of the original polystyrene solution were centrifuged at 7000 rpm for 10 minutes, followed by discarding the supernatant. Later, the polystyrene pellet was dispersed in distilled water and ethanol by sonicating for 1 hour to develop 10 wt.% solution with a suitable distribution of nanospheres. Then, to coat the silk substrates with the hexagonally close packed array of the said nanospheres, a clean 10 mm diameter petri dish was filled with 20-25 mL of distilled water. A glass slide was then placed at a 20° angle to the petri dish. For best results, the wetness of the glass slide was enhanced by 10 minutes of oxygen plasma treatment prior to its usage. A small drop (1 µL) of the said nanoparticle suspension was introduced to the middle of the glass slide. By gliding toward the water surface, the nanospheres were distributed as a monolayer on the water surface. The process was repeated until a complete nanosphere coverage on the surface was attained. Subsequently, ~5 µL of SDS (2% w/v) was introduced to the water to develop a close packed hexagonal array of nanospheres. The monolayer produced at a shallow angle was used to coat the surface of the silk substrates. The coated samples (Figure 4 400) were then placed on another clean container and dried for

further usage. The coated nanospheres on the surface were etched by oxygen plasma for 15 minutes (at 7 mTorr and 50 W) by means of a magnetron sputtering instrument (Phase II J, ATC Orion Sputtering System, AJA International Inc.) rendering the silk substrates nanopatterned structures (nanocones) on the surface (Figures 5A and 5B).

Fabrication of a wound dressing scaffold

To make a wound dressing scaffold, the stretchable silk mesh substrate with nanofeatures on its surface was incorporated in a well-plate and the precursor solution of the BMPS-dACM was cast on it and incubated at -20 °C for 24 hours for its complete gelation.

Bone dECM derived from animal foetus

Foetal cutaneous matrix has the ability to undergo scarless healing in early gestation without any severe inflammation or substantiate fibroplasia. Accordingly, foetal-derived dECM are considered potential candidates for tissue engineering and regenerative medicine applications. Once a foetal tissue is exposed to injury, its response to the damage accompanies larger amounts of glycosaminoglycans (GAGs) such as hyaluronic acid and chondroitin sulfate in comparison with their adult counterparts, enabling facilitated mitosis, migration, and differentiation of the cells. Furthermore, dECM derived from foetal tissues contains larger numbers of immature collagens along with fewer molecular crosslinks compared to the ones derived from their adult counterparts, and thereby undergoes fast degradation and rapid, constructive, and robust remodeling. Accordingly, the distinct regenerative properties and less antigenic nature of the dECM obtained from foetal and/or newborn tissues, compared to their adult equals, make them valuable sources for developing bioactive materials with enhanced cell repopulation yields, substantial regenerative properties, and constructive tissue remodeling. Therefore, bone dECM derived from foetal goat bone is utilized in the claimed invention to fabricate anti-bacterial membranes that are able to substantially regenerate bone while protecting the tissue from possible infection at the site of injury.

Decellularizing foetal goat bone

The frozen foetal goat limbs were thawed overnight at 4°C to separate the bones from muscle, skin, fat, and cartilage. Both the cortical and cancellous bones from femora and tibiae were used for decellularization (Figure 26A). To this end, the bones were cut into small pieces using bone cutters and surgical tools in aseptic conditions (Figure 26B). Next, the frozen bone pieces were ground into granules (Figure 26C). The blood residue were then washed away several times using sterilized MilliQ H₂O. After soaking the bone granules in a sterile hypotonic buffer (10 mM Tris-HCL, pH 8.0) for 1 h, the granules were undergone several freeze-thaw cycles which burst the cells and enhanced cell membrane rupture. Next, the washing buffer was changed to a Triton X-100 (1%) buffer containing protease inhibitor cocktail to remove the remaining blood and cellular compartments efficiently for overnight. Subsequently, granules were washed with sterile MilliQ H₂O until the detergent was removed completely. Then, the samples were incubated with a hypertonic buffer Tris-HCL buffer for up to 2 hours followed by treating with DNase I (50 U/mL) and RNase A (1 U/mL) at 37°C for at least 4 hours. Following that, the samples were washed overnight with the hypotonic buffer added with protease inhibitor cocktail to remove cell remnants and the nucleases used in the previous step. The bone powders were then washed with autoclaved MilliQ H₂O for several times before snap freezing in liquid nitrogen for freeze drying process. The resultant foetal goat bone dECM was kept at -80 °C for further use (Figure 26D). All the washing buffers were supplemented with 1% antibiotic (PSN or PS) except the last cycle of washing with MilliQ H₂O and the samples were shaken during washing steps to facilitate the decellularization process.

Making demineralized foetal goat bone dECM

The frozen foetal goat limbs were thawed overnight at 4°C to separate the bones from muscle, skin, fat, and cartilage. Both the cortical and cancellous bones from femora and tibiae were used for decellularization. The bones were cut into small pieces using bone cutters and surgical tools in aseptic conditions. Next, the frozen bone pieces were ground into granules using a grinder. The blood residue in foetal goat bone granules were then washed away using sterilized MilliQ H₂O supplemented with 1% PSN. The samples were shaken for 50 minutes at room temperature and 250 rpm. The supernatant was discarded and the washing medium was refreshed for several times during this step. Following this, the bone granules were demineralized using sterile 10% EDTA in PBS while being kept on a shaker for 24 hours at 150 rpm at room temperature. After discarding the previous buffer, the bone samples were washed with autoclaved MilliQ H₂O, followed by 2 hour incubation with a hypotonic buffer (10 mM Tris-HCL, pH 8) supplemented with 1% PSN under shaking at room temperature. Subsequently, the washing buffer was refreshed, and the samples exposed to freeze-thaw cycles for several times and the buffer was exchanged between each cycle. Next, the washing buffer was changed to 10 mM Tris-HCL (pH 7.5) supplemented with 3.5% Triton X-100, 1% PSN and protease inhibitor cocktail (2.95 µl/mL). The samples were incubated in a shaking incubator at 37°C and 250 rpm for 4 hours. Subsequently, the detergent was washed away by four cycles of washing with autoclaved H₂O until no bubbles were detected in the samples.

Later, the washing buffer was changed to 50 mM Tris-HCL, 1.5 M NaCl, pH 7.6 and the samples were kept under agitation for 30 min at room temperature. Then, the supernatant was discarded, and the new washing buffer comprises of 10 mM Tris-HCL, pH: 7.5 supplemented with 1% PSN, DNase I (50 U/mL) and RNase A (1 U/mL) was added to the bone granules. This washing step lasted 5 h under agitation at 37 °C. Following that, a hypotonic buffer (10 mM Tris-HCL, pH: 8) added with protease inhibitor cocktail (2.95 µl/mL) and 1% PSN was exploited to remove cell remnants and the nucleases used in the previous step. This cycle was continued for 20 hours at 37°C. Following this, the bone powders were washed with autoclaved MilliQ H₂O up to 72 hours while the samples were shaken at 37°C and the washing medium was refreshed frequently during this step. The decellularized bone granules were snap frozen in liquid nitrogen and kept at -80 °C overnight followed by freeze drying. The achieved demineralized foetal goat bone dECM was kept at -80 °C for further use.

In order to solubilize the demineralized foetal goat bone dECM, the powder was digested in pepsin (2 mg/mL) acidic solution (0.01 M HCL) for 96 hours. The concentration of demineralized foetal goat bone dECM powder in the acidic solution was adjusted to 10-11 mg/mL and kept at 4 °C until use.

Fabrication of dECM-silk fibroin films

To fabricate bone dECM-based membranes, silk fibroin was used as one of the 5 grams of the silkworm (*Bombyx mori*) cocoons were cut and boiled in water (2 L) supplemented with sodium carbonate (Na₂CO₃, 0.02 M) for 30 minutes to degum the silk. Then, the resulting silk was washed with ultrapure water three times followed by submerging it in a LiBr solution (9.3 M) to dissolve completely. Next, the obtained dissolved silk solution was dialyzed against ultrapure water for 48 hours. Subsequently, the attained solution was centrifuged twice at 10000 rpm at 4°C for 20 minutes to eliminate the impurities. It was then blended with the digest of demineralized foetal goat bone dECM upon neutralizing its pH to 7.4 using NaOH (1 M) in different volume ratios of the silk fibroin solution to the said bone digest including 4:1, 3:2, 1:1, 2:3, 1:4 ratios. Subsequently, the said blends (8 mL of each) were cast in petri dishes (60 mm) and left under a range hood until dried completely. Next, the membranes were peeled off from the moulds and water annealed for crosslinking. The achieved dried membranes were named "non-reinforced foetal bone dECM-silk membranes". The optimized ratio of the said blend (1:1) was used to further develop hybrid composite membranes using foetal goat bone dECM. Various amounts of

bone dECM powder (1, 2 or 3 %w/v) with sizes equal or less than 200 μm were mixed with the said blend using a vortex mixer prior to casting. The same procedure mentioned for making non-reinforced bone dECM-silk membrane was subsequently utilised. The resulting membranes were named “foetal bone dECM-silk hybrid composite membranes”.

Developing nanopatterns on the dECM-silk membranes

Colloidal lithography was performed on the water annealed foetal bone dECM-silk membranes with the best mechanical properties (non-reinforced foetal bone dECM-silk membranes with 1:1 ratio of the silk to dECM bone digest which was noted as “non-reinforced dECM-silk (1:1)” and foetal bone dECM-silk hybrid composite membrane containing 2 %w/v dECM bone particles which was noted as “2% dECM-silk hybrid composite”). For this purpose, 2.5 w/v% solution of polystyrene nanospheres (from Tianjin Baseline Chrom Tech Research Center (Product No. 6-1-0030), China) with a size of 300 nm were used. A few drops of the original polystyrene solution were centrifuged at 7000 rpm for 10 minutes and the supernatant was discarded. Later, the polystyrene pellet was dispersed in distilled water and ethanol by sonicating for 1 hour to develop 10 wt.% solution with a suitable distribution of nanospheres. Then, to coat the silk substrates with the hexagonally closely packed array of nanospheres, a clean 10 mm diameter petri dish was filled with 20-25 mL of distilled water. A glass slide was then placed at a 20° angle to the petri dish. For best results, the wettability of the glass slide was enhanced by 10 minute oxygen plasma treatment prior to its usage. A 1 μL drop of the nanoparticle suspension was introduced to the middle of the glass slide. By gliding toward the water surface, the nanospheres were distributed as a monolayer on the water surface. The said process was repeated until complete nanosphere coverage on the surface was attained. Subsequently, ~5 μL of SDS (2% w/v) was introduced to the water to develop a closely packed hexagonal array of nanospheres. The monolayer produced at a shallow angle was used to coat the surface of the silk substrates. The coated samples were then placed in another clean container and dried for further usage. The coated nanospheres on the surface were etched by oxygen plasma for 15 minutes (at 7 mTorr and 50 W) by means of a magnetron sputtering instrument (Phase II J, ATC Orion Sputtering System, AJA International Inc.) rendering the silk substrates’ nanopatterned structures (nanocoons) on the surface.

The experiments as described below provide further examples of the invention as claimed as a dECM-based composition with antibacterial properties for application in tissue regeneration and wound healing. The claimed invention advantageously boosts the healing of tissues that need repair whilst reducing the change of post-operative infections at the site of injury.

EXAMPLE I - DEVELOPING A dECM-BASED WOUND DRESSING SCAFFOLD WITH ANTI-BACTERIAL PROPERTIES WITH POTENTIAL APPLICATION FOR PROMOTING HEALING OF CHRONIC WOUNDS IN DIABETIC MELLITUS

Experiments were designed to fabricate an anti-bacterial wound dressing composite with high potential to be used in clinical applications for promoting chronic wound healing in diabetic mellitus.

An embodiment of the claimed invention includes the decellularization of goat foetal membrane to make soluble dECM powder/granules, functionalizing chitosan amine chains with maleimidyl groups with improved anti-bacterial resistance compared to pristine chitosan, and fabrication of nanopatterned stretchable silk fibroin substrate.

DNA Content Analysis

The residual DNA in the ACM dECM obtained from the method of Example 1 was quantified and compared to that of the non-decellularized tissue as control. The samples were digested at 56 °C using the digestion buffer and proteinase K provided in a DNA extraction kit (DNeasy Blood & Tissue Kit, QIAGEN). Subsequently, the DNA in the digested tissues were purified using the kit according to the manufacturer’s protocol and the achieved purified DNA of each sample was measured by Qant-iT™ PicoGreen® dsDNA Quantification kit in accordance with the provided protocol. The experiments were performed in triplicate. The results, shown in Figure 7 700, confirmed that the DNA content of the dECM was significantly reduced compared to the control ACM by 99.62%. Thus, they are rendered acellular ACM ECM, devoid of cellular debris.

In order to confirm the effective cell removal and collagen preservation in the dECM of ACM, hematoxylin and eosin (H & E) staining and trichrome staining were conducted on the samples produced by the decellularization method of Example 1 and the stained sections were evaluated by light microscopic observation. In black and white images of H & E staining, the nuclei appear in black dots, while the cytoplasm and ECM appear in grey as illustrated in Figures 8A 800 and 8B 810. In black and white images of trichrome staining the fibres of collagen appear in grey, while the nuclei appear in black dots (Figures 9A 900 and 9B 910). As shown in Figures 8A 800-8B 810 and 9A 900-9B 910, the decellularization method of Example 1 can eliminate the cells from the ACM structure considerably well, leaving the collagen well-preserved.

Hydroxyproline assay

The total collagen content of the control and the decellularized foetal goat ACM were quantified using the hydroxyproline assay kit (ab222941) according to the manufacturer’s protocol. Results showed that the collagen content of the ACM had not changed significantly upon using the decellularization method of Example 1, as illustrated in Figure 10 1000.

Atomic Force Microscopy (AFM) Analysis

Compressive elastic modulus of the stretchable silk substrates of the Example 1 were measured according to the force-displacement curves achieved from atomic force microscopy (AFM) analysis by means of Hertz model as previously described. AFM investigations were conducted in triplicate on the samples kept in a desiccator or in normal atmospheric pressure (non-desiccated). The optimal sample used for making the final wound dressing scaffold (containing 3% CaCl_2 to formic acid ratio) was also tested in a wet state to evaluate its mechanical properties under wet/moist conditions which is more relevant to its real application on the wound. Results shown in Figures 11A 1100 and 11B 1110 suggest that the stretchable silk substrates have higher compressive elastic modulus if kept in the desiccator compared to normal atmospheric pressure. Besides this, increasing the CaCl_2 to formic acid ratio in the substrates reduced the compressive elastic modulus considerably. Furthermore, the wetted silk substrate containing 3% CaCl_2 to formic acid ratio with and without nanocones on the surface showed compressive elastic modulus of 4.374 ± 0.28 and 0.63692 ± 0.10 MPa, respectively, as shown in Figure 12 1200. This indicates that the stiffness of the polymeric membranes is reduced once kept in a wet condition and they become softer. However, introducing nanocones on the surface enhances the elastic modulus due to more CaCl_2 removal from the surface leading to a stiffer substrate.

Bacteria Studies

In vitro anti-bacterial properties

Two bacteria strains, *S. aureus* (ATCC 29213) and *Pseudomonas aeruginosa* (*P. aeruginosa*) were used in these assessments. The bacteria were cultured in the lysogeny broth (LB) medium in a shaking incubator at 220 rpm, 37 °C overnight. The bacteria optical density (OD) was adjusted to 0.1 (OD₆₀₀ = 0.1) by supplementing suitable fresh medium which was reactivated for another 3 hours. To assess the anti-bacterial properties of the samples, the ACM dECM powders and the silk substrates were UV-sterilized before the experiments while the chitosan hydrogels were sterilized by passing them through 0.22 µm filters. Next, the sterilized samples including CS, BMPS, BMPS-dACM, silk and nanopatterned silk substrates (both of which made of 3% CaCl₂ to formic acid ratio) were used to test the anti-bacterial effects of oligochitosan, chitosan βMPS functionalization, ACM dECM, silk as well as nanopatterned silk against the said microorganisms. To this end, the sterilized said samples were incorporated in 24-well plates, and an amount of 100 µL of the diluted bacteria solution at a concentration of 10⁵ CFU mL⁻¹ was cultured on the surface of each sample for 1, 3, 6 and 24 hours. Meanwhile, the gaps between the well-plate wells were filled with sterile water to avoid evaporation of the culture medium. At the above timepoints, the attached bacteria were detached from the samples by adding 900 µL of the medium to each well and by pipetting up and down. Next, appropriate dilutions of the said bacteria suspension were used for each time point and spread on a solid agar plate and cultured for another 16 hours to count the correlated CFU. The anti-bacterial rates of the samples were determined by the following formula:

$$\text{Antibacterial rate(\%)} = 1 - \frac{\text{CFU}_{\text{Experimental group}}}{\text{CFU}_{\text{Control group}}}$$

As a control for anti-bacterial activity of the silk or chitosan samples, a well of 24 well-plate was used to culture the bacteria and perform the experiment with the same conditions for each time point.

As illustrated in Figures 13 1300 and 14 1400, the chemical functionalization of oligochitosan with βMPS improves the anti-bacterial efficiency of pristine chitosan hydrogel (CS) against *S. aureus* while incorporation of ACM dECM in the functionalized chitosan gel shows the best anti-bacterial properties. The effect of chitosan βMPS functionalization on enhancing the anti-bacterial properties of pristine chitosan against *P. aeruginosa* can be seen in Figure 15 1500.

Figures 16 1600 and 17 1700 show the photographs of agar plates onto which *S. aureus* and *P. aeruginosa* bacteria were recultivated after treatment with a well of 24-well plate as control, silk and nanopatterned silk for 1, 3, 6 and 24 hours, respectively. Results confirm that the nanocones on the surface of the stretchable silk substrate interact with both *S. aureus* and *P. aeruginosa* significantly and improve the anti-bacterial properties of the pristine silk films. While silk has some anti-bacterial effect against *P. aeruginosa*, introduction of the nanocones on its surface can considerably increase its anti-bacterial rate.

Adhesion Tests On Stretchable Silk Membranes

Stretchable silk membranes made of the same composition (CaCl₂ to formic acid ratio of 3%), with and without nanopatterns were used for bacteria adhesion tests. Samples were UV sterilized before these experiments in a biosafety cabinet. Two bacteria strains, *S. aureus* and *P. aeruginosa* were used in these assessments. The bacteria were cultured in the LB medium in a shaking incubator at 220 rpm, 37 °C overnight. The bacteria optical density was adjusted to 0.1 (OD₆₀₀ = 0.1) by supplementing suitable fresh medium which were reactivated for another 3 hours. To assess the bacteria adhesion, the bacteria concentration was adjusted to 10⁵ CFU mL⁻¹ and 100 µL of the solution was spread on the substrate surfaces to mimic immersion. At different time points (1, 3, 6 and/or 24 hours), the culture medium was removed to fix the substrates with 2.5% glutaraldehyde overnight. Later, the samples were washed successively with 10, 30, 50, 75, and 96% ethanol, and dried at 25 °C before the observation with SEM (Figures 18A-18D, 19A-19D, 20A-20D, 21A-21D). Results confirm that the nanocones on the surface interact with both *S. aureus* and *P. aeruginosa* bacteria, affect their morphology, and inhibit adhesion and formation of biofilm on the nanopatterned surface significantly compared to its counterpart without nanofeatures (silk).

Animal Studies

All experiments were approved by the Animal Research Ethics Committee of the Chinese University of Hong Kong (Ref. No. 22-364-HMF). All db/db mice were purchased from the animal center of the Chinese University of Hong Kong with the animal research ethical approvals. The mice were maintained under controlled temperature around 20 °C with a 12 h light/12 h dark cycle with free access to water and a pelleted commercial diet.

The full-thickness skin defect mouse model was established as followings: 6 weeks old db/db mice (male, average weight 32.2g ± 2.4g) were used with general anesthesia and sterile condition. Two parallel 6 mm circular (surface area 28.26 mm²) full-thickness skin wounds were created on the back of each mouse. A polyethylene collar was stitched to the adjacent skin to retain the margin of each wound.

Animal groups

To detect the effects of different samples on diabetic wound healing post full thickness skin defect surgery, db/db mice were randomly divided into 5 groups (n=6 per group). The wounds were treated with 1) nothing (NC group) or treated with hydrogel scaffolds, 2) CS, 3) BMPS, 4) BMPS-dACM, and 5) ChitoSilkBioPatch. The width of the scaffolds in each group were adjusted according to the wound size diameter (6 mm). The wound images were captured at days 0, 3, 7, 10 and 14 post surgery. The splints were removed 7 days after surgery. The skin wound samples were harvested at days 7 and 14 post surgery for the tissue regeneration assessment by histological analysis and immunofluorescence staining.

Histological analysis

Mice of different groups were sacrificed (7 and 14 days post-surgery) with overdosed and the wound specimens including full thickness skin layers (epidermis, dermis, and hypodermis) were fixed in 4% buffered paraformaldehyde and embedded in paraffin. 7 µm tissue sections were subjected to H&E staining and photographed by Leica image analyzing system. 7 µm tissue sections subjected to Sirius Red staining were photographed by the polarizing microscope. Images were captured under 100x with Leica DM5500 system.

Immunofluorescence staining

7 µm tissue sections were subjected to immunofluorescence double-labeling staining. Briefly, after antigen retrieval, primary antibodies against anti-F4/80 (1:200; ab6640, Abcam, USA), anti-iNOS (1:200; ab15323, Abcam, USA), anti-CD206 (1:200; ab64693, Abcam, USA), anti-CD31 (1:200; ab182981, Abcam, USA), anti-α-SMA (1:200; ab7817, Abcam, USA) were used, and tissue sections were incubated with primary antibodies for 16 h at 4°C. This was followed by incubation with secondary antibody Alexa 488 (1:500 diluted; ab150077, Abcam, USA) or Alexa 594 (1:500 diluted; ab150160, Abcam, USA) for 1 h at room temperature. Nucleus were stained with ProLong™ Diamond Antifade DAPI (Life Technologies, USA). Images were captured under 200x with Leica DM5500 system and analyzed with Image Pro Plus 6.0.

Ethical Statement

Animal studies were conducted under the animal license issued by the Hong Kong SAR Government and the approval of the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong (Ref. No. 22-364-HMF).

Statistical Analysis

All the experiments were repeated at least 3 times. The results were presented as mean $\pm$ standard deviation. A single-factor ANOVA was employed to evaluate the statistical significance of the results, with a significance level set at $P < 0.05$ (*).

The biocompatibility and bioactivity of the ChitoSilkBioPatch were evaluated using the said chronic wound model established with db/db mice. Full thickness skin wounds (diameter: 6 mm) were created on the back of the db/db mice and subsequently covered with CS, BMPS, BMPS-dACM, ChitoSilkBioPatch or nothing (Control group, NC). The wound healing outcomes were examined with macroscopic observation and histological evaluation by H&E and Sirius Red staining. Covering with nanopatterned silk meshes, the wound closure condition in the ChitoSilkBioPatch group was hard to observe and record on and before day 7. As the silk meshes shed off synchronizing with wound contraction in the ChitoSilkBioPatch group between day 7 and day 10 post-surgery, the wound closure rates in all groups were calculated on day 10 and day 14 post-surgery (as referred to in Figure 22A).

In comparison with NC, CS and BMPS groups, the wound treated with BMPS-dACM hydrogels and ChitoSilkBioPatch showed significantly better healing outcomes. In BMPS-dACM and ChitoSilkBioPatch groups, a higher healing ratio was observed macroscopically on day 10 and 14; H&E staining (as shown in Figure 22B) revealed better neodermis regeneration and new epidermis formation on day 7 post-surgery, and more complete epithelialization and dermis regeneration on day 14 post-surgery. Sirius Red staining (as shown in Figure 23) demonstrated that tighter and better oriented collagen fibers together with more collagen I formation was also observed in BMPS-dACM and ChitoSilkBioPatch groups on day 7 and 14 post-surgery. These results showed that the utilization of dACM brought notable bioactivities to the bio-patch and significantly facilitated wound healing. Interestingly, from macroscopic observation and histological evaluation, we observed a better healing outcome in ChitoSilkBioPatch group in comparison with BMPS-dACM group. Accelerated wound closure ratio together with enhanced epidermis and dermis regeneration were observed in ChitoSilkBioPatch group. The nanopatterned silk mesh itself theoretically possesses limited bioactivity in wound healing, and therefore the enhanced wound healing potentially came from its physical protection of the wound.

Furthermore, in comparison with NC and CS groups, the wound treated with the BMPS hydrogel showed significantly higher macroscopic healing ratio and enhanced quality of epithelialization and dermis regeneration. Results indicate that BMPS presents stronger bioactivities in promoting chronic wound healing than CS hydrogels.

To further explore the potential cellular mechanism of ChitoSilkBioPatch in promoting chronic wound healing, its *in vivo* immunomodulating and angiogenic effects were further evaluated with IF staining (as referenced in Figure 24, 25). Results in Figure 24 demonstrated that on day 7 post-surgery, significantly less iNOS expressing inflammatory macrophages (M1 phenotype) and more CD206+ anti-inflammatory macrophages (M2 phenotype) were found in BMPS-dACM and ChitoSilkBioPatch groups, compared to NC, CS and BMPS groups, demonstrating that the incorporation of dACM brought desirable immunomodulating effects to ChitoSilkBioPatch. Furthermore, BMPS hydrogels presented significantly stronger immunomodulating effects than CS hydrogels. Significantly less M1 macrophages were observed in wounds treated with BMPS hydrogels compared to NC and CS groups. IF staining of CD31 and α -SMA (as shown in Figure 25) showed that the number of neocapillaries in the regenerated tissues of BMPS-dACM and ChitoSilkBioPatch groups was significantly larger than that in the NC, CS and BMPS groups.

The above results revealed desirable biocompatibility and bioactivity of ChitoSilkBioPatch. No obvious adverse events or reactions were observed in either group. The superior bioactivities of ChitoSilkBioPatch mainly stem from dACM and also partially from BMPS. As a highly biocompatible natural scaffold as well as a source of several potent GFs, cytokines and matrix proteins, the ACM derivatives have been studied and applied for decades and reported to present various therapeutic advantages in wound healing. AM offers various biological effects including promotion of epithelialization, modulation of angiogenesis, inhibition of inflammation and fibrosis, and antimicrobial properties. Here, dACM supplementation into the bio-patch brought notable bioactivities and significantly facilitated wound healing. Accelerated epidermis and dermis regeneration together with enhanced angiogenic and immunomodulating effects were observed in BMPS-dACM and ChitoSilkBioPatch groups. Considering the removal of cellular components, the bioactivities of dACM in our study are presumably attributed to the presence of GFs, cytokines, and other bioactive proteins. In diabetic wounds, the alterations of the involved cellular profile and function contribute to development of chronic healing. Intense and prolonged inflammation is a dominant feature affecting the process of diabetic wound healing (see G. F. Pierce, "Inflammation in nonhealing diabetic wounds: The space-time continuum does matter," *Am. J. Pathol.*, vol. 159, no. 2, pp. 399–403, 2001, doi: 10.1016/S0002-9440(10)61709-9.). The excessive accumulation of pro-inflammatory macrophages and aberrant polarization of macrophage play a pivotal role in diabetic wounds healing.

In comparison with CS, BMPS demonstrated promoting effects in wound healing and immunomodulation, making it more desirable as a scaffold for chronic wound repair. Chitosan and its derivatives exert many biological activities including antimicrobial and anti-inflammatory activities, which has been studied for decades (see P. Feng et al., "Chitosan-Based Functional Materials for Skin Wound Repair: Mechanisms and Applications," *Front. Bioeng. Biotechnol.*, vol. 9, no. February, 2021, doi: 10.3389/fbioe.2021.650598.). However, these activities are considered relatively mild necessitating chitosan modification. In this study, stronger anti-inflammatory activities were demonstrated compared with CS, which is possibly attributed to the grafted maleimide components as maleimide and its derivatives have been reported to present anti-inflammatory effects (see Y. Zhang et al., "Design, synthesis and biological evaluation of novel chromone-maleimide hybrids as potent anti-inflammatory agents against LPS-induced acute lung injury," *Bioorg. Chem.*, vol. 128, no. March, p. 106049, 2022, doi: 10.1016/j.bioorg.2022.106049. and S. D. Firke and S. B. Bari, "Synthesis, biological evaluation and docking study of maleimide derivatives bearing benzenesulfonamide as selective COX-2 inhibitors and anti-inflammatory agents," *Bioorganic Med. Chem.*, vol. 23, no. 17, pp. 5273–5281, 2015, doi: 10.1016/j.bmc.2015.07.070.). Furthermore, the antibacterial properties of BMPS demonstrated in our *in vitro* studies may also contribute to the downregulated inflammatory response in the wound. In summary of animal study findings, BMPS demonstrated stronger anti-inflammatory and regenerative bioactivities than CS, making it more desirable in chronic wound repair. The addition of dACM brought notable bioactivities including accelerating epidermis and dermis regeneration and enhancing angiogenesis and immunomodulation, and significantly facilitated wound healing. Application of nanopatterned silk mesh onto the wound didn't impede wound healing. Moreover, enhanced wound healing was observed in ChitoSilkBioPatch group compared with BMPS-dACM, which potentially came from the physical protection of nanopatterned silk mesh for the wound. Our proposed biopatch demonstrated desirable bioactivities including antibacterial, anti-inflammatory and pro-angiogenic effects, presenting great therapeutic potential in chronic wound healing.

EXAMPLE II —DEVELOPING ANTI-BACTERIAL POLYMERIC MEMBRANES BASED ON FETAL BONE DECM WITH POTENTIAL APPLICATION AS ANTI-BACTERIAL BONE GUIDANCE FOR REGENERATION OF BONE LESIONS

Experiments were designed to fabricate anti-bacterial membranes for bone regeneration purposes. The membranes thus have high potential to be used in clinical applications as bone guidance wherein the tissue/lesion is susceptible to bacterial infections.

Embodiments of the claimed invention include the decellularization of xenogeneic foetal derived bone ECM to produce both mineralized and demineralized foetal goat bone dECM powder/granules, silk fibroin and a combination thereof, and fabrication of nanopatterned xenogeneic foetal bone dECM-based membrane.

DNA content analysis

The residual DNA in both foetal goat bone dECM and demineralized foetal goat bone dECM obtained from the method of Example 2 was quantified and compared to that of the non-decellularized tissue as control. The samples were digested at 56 °C using the digestion buffer and proteinase K provided in a DNA extraction kit (DNeasy Blood & Tissue Kit, QIAGEN). Subsequently, the DNA in the digested tissues were purified using the kit according to the manufacturer's protocol and the purified DNA of each sample was measured by Qant-iT™ PicoGreen® dsDNA Quantification kit according to the provided protocol (Figure 27 2700). The experiments were performed in triplicate. The results confirmed that the DNA contents of both foetal goat-derived bone dECM and demineralized bone dECM were significantly reduced compared to the control tissue by 99.46% and 99.84%, respectively. Thus, they can be considered acellular ECM, devoid of cellular debris.

Histological analysis

In order to confirm the effective cell removal and collagen preservation in the dECM of foetal goat bone samples, H & E staining and trichrome staining were conducted on the samples produced by the method of Example 2 and the stained sections were evaluated by light microscopic observation. In black and white images of H & E staining, the nuclei appear in black spots, while the cytoplasm and ECM appear in grey, as illustrated in Figures 28A-28C 2800, 2810, 2820. In black and white images of trichrome staining the fibres of collagen appear in grey, while the nuclei appear in black spots (Figures 29A-29C). As shown in Figs 28A-28C, 29A-29C 2900 2910 2920, both of the decellularization methods of Example 2 considerably eliminate the cells from the tissue structure while ensuring the collagen is well-preserved.

Scanning electron microscopy (SEM) evaluations

The morphology of the dECM-silk based membranes produced by the method of Example 2 were studied under SEM. The SEM images indicated that addition of the silk fibroin to the bone demineralized dECM digest led to the formation of microfibers in the substrate structure. Furthermore, the increment in the silk fibroin content resulted in larger microfibers in non-reinforced foetal bone dECM-silk membranes. Meanwhile the sample with the highest silk concentration showed an equiaxed granular-like structure full of cracks leading to a much more brittle platform. The said sample was too brittle to be able to handle and peel off successfully from the mould (Figure 30 3000). The morphology of the nanopatterned non-reinforced dECM-silk (1:1) shown in Figure 31A 3100 confirms the formation of hexagonally closely packed nanocones on the surface which are made of both demineralized dECM and silk fibroin. Additionally, the nanocones are also expressed on the surface of the microfibers in the membrane, rendering a uniform distribution of microfibers with hexagonally close packed arrays of nanocones on the substrate surface.

Atomic force microscopy (AFM) analysis

Compressive elastic modulus of the dECM-silk films of Example 2 were measured according to the force-displacement curves achieved from AFM analysis by means of Hertz model as previously described. AFM investigations were conducted in triplicate. It was seen that compressive elastic modulus of the non-reinforced foetal bone dECM-silk membranes was enhanced by increasing the volume ratio of the silk fibroin solution in relation to the demineralized bone dECM digest. The elastic modulus of the sample with 20% silk of 1.34±0.1 GPa was increased to 1.46±0.2 GPa once the silk concentration enhanced to 80%. However, the samples with higher silk content were more brittle and the best sample in terms of handleability and mechanical performance contained 50% silk fibroin with 1.35±0.05 GPa compressive elastic modulus. The non-reinforced dECM-silk substrate was further reinforced with 1, 2 and 3 % w/v bone dECM powder which led to the enhancement of its elastic modulus to 1.39±0.5, 1.54±0.5 and 1.63±0.09 GPa, respectively.

The morphology of the 2% dECM-silk hybrid composite and its nanopatterned counterpart are shown in Figures 32A-32D (3200, 3210, 3220, 3230). The yellow arrows indicate the dispersion of foetal bone dECM particles with sizes ≤ 200 µm in the substrate.

Bacteria studies

In vitro anti-bacterial properties

Bacterial strain, *S. aureus* (ATCC 29213) was used in these assessments. The bacteria were cultured in the LB medium in a shaking incubator at 220 rpm at 37 °C overnight. The bacteria optical density was adjusted to 0.1 (OD₆₀₀ = 0.1) by supplementing suitable fresh medium which was reactivated for another 3 hours. To assess the anti-bacterial properties of the samples, the membranes were UV-sterilized before the experiments. The O₂ plasma-etching was performed for 5 minutes on non-reinforced dECM-silk (1:1) and 2% dECM-silk hybrid composite to increase the hydrophilicity of their surfaces for subsequent in vitro studies. Thus, the sterilized samples included well plate as negative control, O₂ plasma-etched non-reinforced dECM-silk (1:1), nanopatterned non-reinforced dECM-silk (1:1), O₂ plasma-etched 2% dECM-silk hybrid composite, nanopatterned 2% dECM-silk hybrid composite, and ciprofloxacin as a positive control at a concentration of 20 mg/mL were used to test the anti-bacterial properties of the materials against the microorganism. In order to choose the best initial concentration of bacterial solution for this test, nanopatterned 2% dECM-silk hybrid composites were treated with 100 µL of the bacteria solution at different concentrations for 3 hours. A well of a 24 well plate was used as control. The gaps between the well-plate wells were filled with sterile water to avoid evaporation of the culture medium. At the timepoint of 3 hours, the attached bacteria were detached from the samples by adding 900 µL of the medium to each well and pipetting up and down. Next, appropriate dilutions of the said bacteria suspension were used and spread on a solid agar plate and cultured for another 16 hours to count the correlated CFU. Figure 33 3300 confirmed that the nanopatterned 2% dECM-silk hybrid composite has significant anti-bacterial properties even when the concentration of the bacteria solution was adjusted to 10⁹ CFU mL⁻¹. Therefore, to be able to compare the anti-bacterial properties of the samples with each other, a high concentration of *S. aureus* (10⁸ CFU mL⁻¹) was used for the rest of the experiments. To this end, the sterilized samples were incorporated in 24-well plates, and an amount of 100 µL of the diluted bacteria solution at a concentration of 10⁸ CFU mL⁻¹ was cultured on the surface of each sample for 1, 3, 6 and 24 hours. Meanwhile, the gaps between the wells were filled with sterile water to avoid evaporation of the culture medium. At the specified timepoints, the attached bacteria were detached from the samples by adding 900 µL of the medium to each well and pipetting up and down. Next, appropriate dilutions of the said bacteria suspension were used for each time point and spread on a solid agar plate and cultured for another 16 hours to count the CFU.

Figure 34 3400 shows photographs of agar plates onto which *S. aureus* bacterial cells were recultivated after treatment with wells comprising a negative control, O₂ plasma-etched non-reinforced dECM-silk (1:1), nanopatterned non-reinforced dECM-silk (1:1), O₂ plasma-etched 2% dECM-silk hybrid composite, nanopatterned 2% dECM-silk hybrid composite, and ciprofloxacin as a positive control at concentration of (20 mg/mL) for 1, 3, 6 and 24 hours. Surface nanocones of both nanopatterned samples improved the anti-bacterial properties of their counterparts without nanocones (O₂ plasma-etched non-reinforced dECM-silk (1:1) and O₂ plasma-etched 2% dECM-silk hybrid composite) against *S. aureus* considerably, although all the membranes containing foetal bone dECM (mineralized or demineralized) have interesting anti-bacterial properties considering the very high initial concentration of the bacterial solution (10⁸ CFU mL⁻¹). Furthermore, the 2% dECM-silk hybrid composites with or without nanocones showed much better antimicrobial effects against *S. aureus* compared to the non-reinforced membranes.

Adhesion Tests

Silk membranes with and without nanopatterns were used for bacteria adhesion tests. Samples including O₂ plasma-etched non-reinforced dECM-silk (1:1), nanopatterned non-reinforced dECM-silk (1:1), O₂ plasma-etched 2% dECM-silk hybrid composite, nanopatterned 2% dECM-silk hybrid composite were UV sterilized before these experiments in a biosafety cabinet. *S. aureus* was cultured in the LB medium in a shaking incubator at 220 rpm at 37 °C overnight. The bacteria optical density was adjusted to 0.1 (OD₆₀₀ = 0.1) by supplementing suitable fresh medium and reactivated for another 3 hours. To assess the bacteria adhesion, the bacteria concentration was adjusted to 10⁸ CFU mL⁻¹ and 100 µL of the solution was spread on the substrate surfaces to mimic immersion. At different time points (1, 3, 6 and/or 24 hours), the culture medium was removed to fix the substrates with 2.5% glutaraldehyde overnight. Later, the samples were washed successively with 10, 30, 50, 75, and 96% ethanol, and dried at 25°C before the observation with SEM (shown in Figures 35A-35D (3500, 3510, 3520, 3530) and 36A-36D (3600, 3610, 3620, 3630)). It is clear from Figures 35A-35D that the bacteria cell walls have been damaged once cultured in both Figures 35A 3500 to 35B 3510 O₂ plasma-etched non-reinforced dECM-silk (1:1) and Figures 35C 3520 to 35D 3530 nanopatterned non-reinforced dECM-silk (1:1) for 3 hours. This is due to the effective anti-bacterial activity of AMPs which exist in the foetal demineralized bone dECM component of the said membranes. AMPs in the living organisms are produced by their innate immune system to act against fungi, viruses and bacteria. They are typically shorter than 45 amino acids, amphipathic and cationic. These peptides are remarkably diverse in structure, function, and sequence, even between closely correlated species. Such AMPs are able to interact with the negatively charged lipids of bacterial membranes which result in permeabilization and destabilization of the said membranes, along with causing multiple stresses on the bacterial membrane proteins and the leakage of the contents in the bacterial cell as illustrated in Figure 35A 3500. Several mechanisms have been proposed to describe the mode of action of AMPs. For example, some models have been proposed to explain the interaction of α -helical AMPs with the bacteria membranes, such as "toroidal pore," "barrel stave," and "carpet model". All the mechanisms cause severe damage of bacterial membrane leading to bacterial death. As can be seen in Figures 35A-35D (3500, 3510, 3520, 3530), the holes located at the bacterial cell walls confirm the severe damage to the membranes which inhibit the bacterial growth.

The peptides might be derived from collagenous protein, non-collagenous protein, and growth factors. Also, the degraded products of ECM components bring antimicrobial activity since most of them have hydrophobic and basic amino acid sequences leading to anti-bacterial activities against Gram-positive and Gram-negative bacteria. Some AMPs are cationic and since the bacterial membrane is highly negative due to its surface components, they will interact with each other, and thus the electrostatic forces will influence the anti-bacterial activity of the AMPs.

Although both the samples have anti-bacterial effects against *S. aureus*, the nanopatterned sample has a smaller number of the bacteria on its surface (Figures 35C 3520, 35D 3530) compared to its counterpart without nanostructures (Figures 35A 3500-35B 3510). This can be due to the synergistic anti-bacterial effects of the nanocones which interact with cell walls and the dECM AMPs of the foetal demineralized bone used in the substrate of nanopatterned non-reinforced dECM-silk (1:1) leading to considerably less bacterial attachment to the surface.

SEM images of *S. aureus* bacterium cultured on (Figures 36A 3600-36B 3610) O₂ plasma-etched 2% dECM-silk hybrid composite and nanopatterned 2% dECM-silk hybrid composite for 3 hours (Figures 36C 3620-36D 3630) confirm the anti-bacterial properties of both samples as the bacteria cell walls have been damaged when cultured on these surfaces. However, the synergistic effects of both the nanocones which interact with cell walls and the dECM AMPs of the foetal bone (mineral and demineralized) used in the substrate of the nanopatterned 2% dECM-silk hybrid composite caused less bacterial attachment to the surface with more significant bacterial membrane damage. While some holes are created on the bacterial cell walls in the O₂ plasma-etched 2% dECM-silk hybrid composite, more severe damage can be detected on the cell wall of the bacteria cultured on the nanopatterned surface, with more bacterial debris vividly seen in the SEM image of the nanopatterned 2% dECM-silk hybrid composite (Figures 36C 3620-36D 3630).

Comparing Figure 35D 3530 with Figure 36D 3630, the existence of more AMPs in the nanopatterned 2% dECM-silk hybrid composite due to the incorporation of bone dECM particles (other than the demineralized bone dECM digest in the substrate) and the resultant stiffer nanocones on its surface has a synergistic anti-bacterial effect and thus causes more severe bacterial cell wall damage compared to the nanopatterned non-reinforced dECM-silk (1:1).

Figures 37A-37D (3700, 3710, 3720, 3730) illustrate the SEM images of *S. aureus* bacterium cultured on O₂ plasma-etched non-reinforced dECM-silk (1:1) and nanopatterned non-reinforced dECM-silk (1:1) for 24 hours confirming the damage of the bacterial cell walls on both samples up to 24 hours. However, the synergistic effects of both the nanocones and the dECM AMPs of the foetal demineralized bone used in the substrate of the nanopatterned non-reinforced dECM-silk (1:1) caused less bacterial attachment to the surface or more anti-bacterial activity compared with its counterpart without nanopatterns. Figures 38A-38D (3800, 3810, 3820, 3830) show the SEM images of *S. aureus* bacterium cultured on O₂ plasma-etched 2% dECM-silk hybrid composite and nanopatterned 2% dECM-silk hybrid composite for 24 hours, confirming the anti-bacterial properties of both substrates as the bacteria cell walls have been damaged significantly. However, the synergistic effects of both the stiff nanocones and the dECM AMPs of the foetal bone (mineral and demineralized) used in the substrate of the nanopatterned 2% dECM-silk hybrid composite caused less bacterial attachment to the surface with more severe bacteria membrane damage. Almost none of the bacteria on the surface are alive and only bacteria debris can be seen on this substrate. Comparing Figure 37D 3730 with Figure 38D 3830, the existence of more AMPs in the nanopatterned 2% dECM-silk hybrid composite due to the incorporation of bone dECM particles (other than the demineralized bone dECM digest in the substrate) and the resultant stiffer nanocones on its surface has a synergistic anti-bacterial effect and thus causes more severe bacterial cell wall damage compared to the nanopatterned non-reinforced dECM-silk (1:1). Although the bacteria cell walls have been damaged on both surfaces, the damage caused by the nanopatterned 2% dECM-silk hybrid composite is more significant as only cell debris can be detected on this surface while rounded bacteria with obvious holes in their membranes can be seen on the surface of its counterpart without the bone dECM particles.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Any reference to prior art contained herein is not to be taken as an admission that the information is common general knowledge, unless otherwise indicated.

CLAIMS:

1. A composition for tissue regeneration in a subject, comprising a xenogeneic decellularized extracellular matrix (dECM) component containing a biological material, and a polymeric component, wherein the composition is produced by a method comprising:
 - (i) isolating tissue from a donor animal to obtain the biological material;
 - (ii) decellularizing the biological material to obtain the dECM component; and
 - (iii) incorporating the dECM component with the polymeric component to produce the composition having enhanced antibacterial properties.
2. The composition of claim 1, wherein the tissue is selected from one or more of foetal membrane or foetal or neonatal bone from the donor animal.
3. The composition of claim 1, wherein the donor animal is a foetal or neonatal goat.
4. The composition of claim 1, wherein the donor animal is a foetal or neonatal goat with a gestational age of about 12 weeks to about 18 weeks.
5. The composition of claim 1, wherein the tissue is selected from one or more of foetal membrane tissue and/or foetal or neonatal bone tissue from the donor animal.
6. The composition of claim 5, wherein the tissue is a foetal membrane tissue comprising one or more of amnion and/or chorion membranes.
7. The composition of claim 1, wherein the tissue is foetal or neonatal bone tissue comprising one or more of cortical bone and/or cancellous bone.
8. The composition of claim 1, wherein the dECM component is in the form of a powder, solution, hydrogel, scaffold, membrane, film, coating, or a combination thereof.
9. The composition of claim 1, wherein a surface morphology of the dECM component is porous, smooth, nanopatterned, or a combination thereof.
10. The composition of claim 1, wherein the polymeric component is derived from one or more natural polymers.
11. The composition of claim 1, wherein the polymeric component is silk fibroin, chitosan, or a combination thereof.
12. The composition of claim 1, wherein the polymeric component is silk fibroin in a stretchable film or a stiff film form.
13. The composition of claim 1, wherein the polymeric component is pure chitosan or a functionalised chitosan.
14. The composition of claim 1, wherein the polymeric component is functionalised chitosan created by mixing chitosan with N-(β -maleimidopropoxy) succinimide ester for about 6 hours at room temperature.
15. The composition of claim 1, wherein the polymeric component is a film, scaffold, hydrogel, or a combination thereof.
16. The composition of claim 1, wherein a surface morphology of the polymeric component is porous, smooth, nanopatterned, or a combination thereof.
17. The composition of claim 1, wherein a nanopatterned surface morphology of the dECM component and/or the polymeric component provides the composition with enhanced antibacterial properties.
18. The composition of claim 1, wherein the composition is a scaffold, membrane, or a coating.
19. The composition of claim 1, wherein the composition is applied to skin or bone tissue of a subject where tissue repair is required.
20. A method of producing a decellularized extracellular membrane (dECM) composition comprising a dECM component and a polymeric component for tissue regeneration in a subject, comprising the steps of:
 - (i) obtaining tissue of interest from a donor animal;
 - (ii) decellularizing the tissue of interest and removing genetic material from the tissue of interest to obtain the dECM component; and
 - (iii) incorporating the dECM component with the polymeric component to produce the composition having enhanced antibacterial properties.
21. The method of claim 20, wherein decellularizing the tissue of interest comprises treating the tissue of interest with chemical agents, including detergents, enzymes, chelating agents, or a combination thereof.
22. The method of claim 20, wherein decellularizing the tissue of interest includes the following steps:
 - (i) washing the tissue in a hypotonic buffer, optionally supplemented with an antibiotic, a chelating agent, and a protease inhibitor cocktail;
 - (ii) washing the tissue in an isotonic buffer, optionally containing a detergent, a chelating agent, a protease inhibitor cocktail, and an antibiotic;
 - (iii) washing the tissue with the isotonic buffer;
 - (iv) washing the tissue in the hypotonic buffer; and
 - (v) washing the tissue with the isotonic buffer and removing genetic material from the tissue of interest, resulting in the dECM component.
23. The method of claim 20, wherein decellularizing the tissue of interest includes an optional step of physically disrupting the tissue of interest by freeze-thawing, grinding, homogenization, osmotic shock, or a combination thereof.
24. The method of claim 20, wherein the polymeric component is derived from one or more natural polymers.
25. The method of claim 20, wherein the polymeric component is silk fibroin, chitosan, or a combination thereof.

26. The method of claim 20, wherein the polymeric component is silk fibroin in a stretchable film or a stiff film form.
27. The method of claim 20, wherein the polymeric component is pure chitosan or a functionalised chitosan.
28. The method of claim 20, wherein the polymeric component is functionalised chitosan created by mixing chitosan with N-(β -maleimidopropoxy) succinimide ester for about 6 hours at room temperature.
29. The method of claim 20, wherein the polymeric component is a film, scaffold, hydrogel, or a combination thereof.
30. The method of claim 20, wherein a surface morphology of the polymeric component is porous, smooth, nanopatterned, or a combination thereof.
31. The method of claim 20, wherein a nanopatterned surface morphology of the dECM component and/or the polymeric component provides the dECM composition with enhanced antibacterial properties.
32. The method of claim 20, wherein the dECM component is in the form of a powder, hydrogel, scaffold, membrane, film, coating, or a combination thereof.
33. The method of claim 20, wherein a surface morphology of the dECM component is porous, smooth, nanopatterned, or a combination thereof.
34. The method of claim 20, wherein the dECM composition is a scaffold, membrane, or a coating.
35. The method of claim 20, wherein the donor animal is a foetal or neonatal goat.
36. The method of claim 20, wherein the tissue of interest is selected from one or more of foetal membrane or foetal or neonatal bone from the donor animal.
37. The method of claim 20, wherein the donor animal is a foetal or neonatal goat with a gestational age of about 12 weeks to about 18 weeks.
38. The method of claim 20, wherein the tissue of interest is selected from one or more of foetal membrane tissue and/or foetal or neonatal bone tissue from the donor animal.
39. The method of claim 20, wherein the tissue of interest is foetal membrane tissue comprising one or more of amnion and/or chorion membranes.
40. The method of claim 20, wherein the tissue of interest is foetal or neonatal bone tissue comprising one or more of cortical bone and/or cancellous bone.
41. The method of claim 20, wherein the dECM composition is applied to skin or bone tissue of the subject where tissue repair is required.
42. A method of repairing skin or bone tissue in a subject, the method comprising applying the dECM composition of claim 20 to the skin or bone tissue of the subject where tissue repair is required.

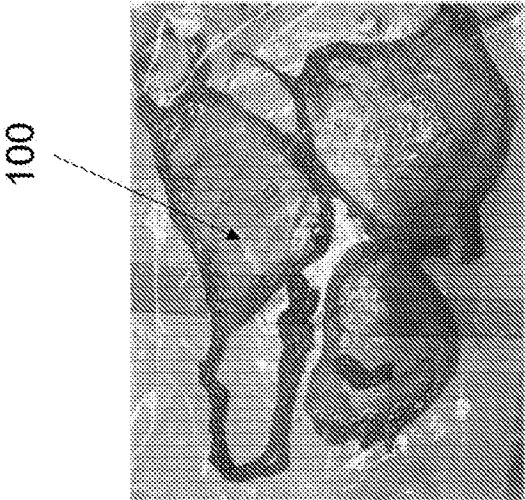


Figure 1C

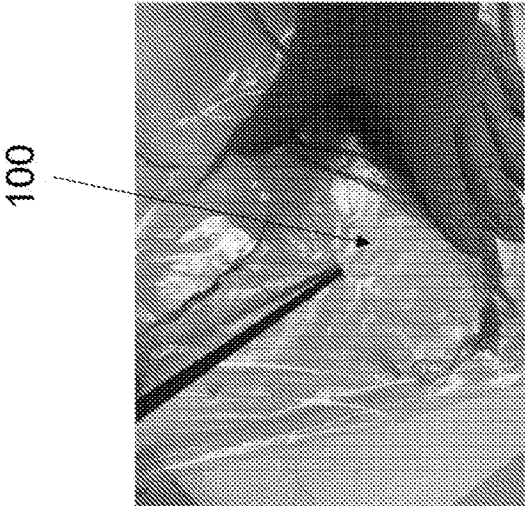


Figure 1B

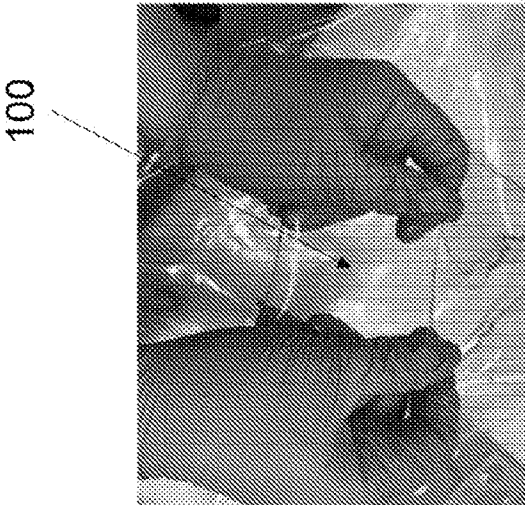


Figure 1A

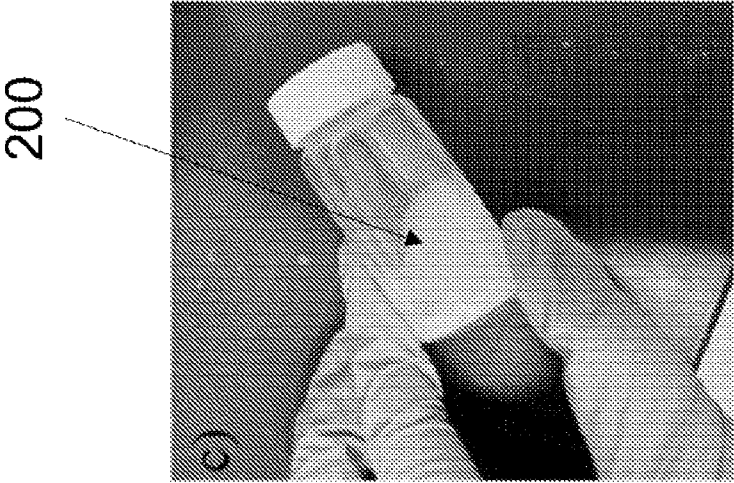


Figure 2C

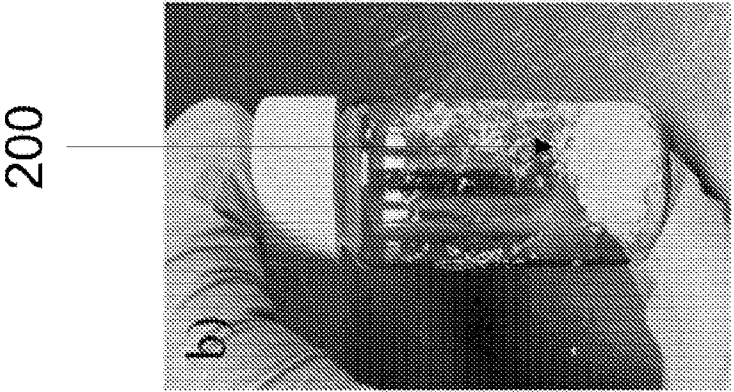


Figure 2B

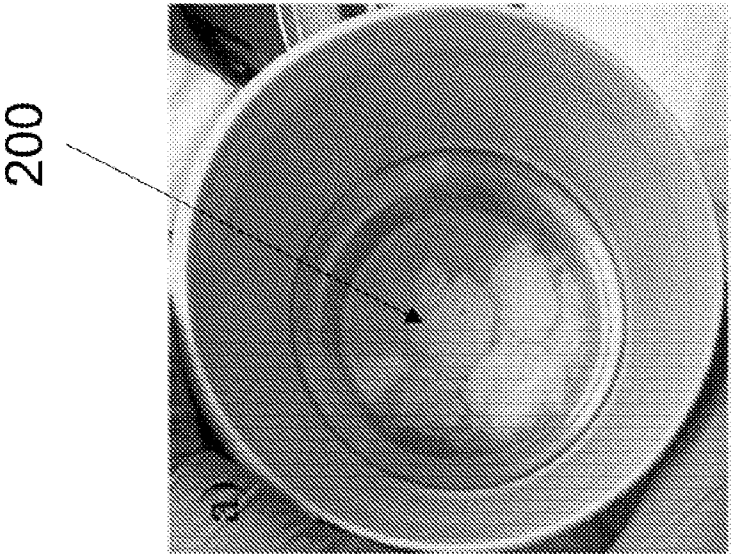


Figure 2A

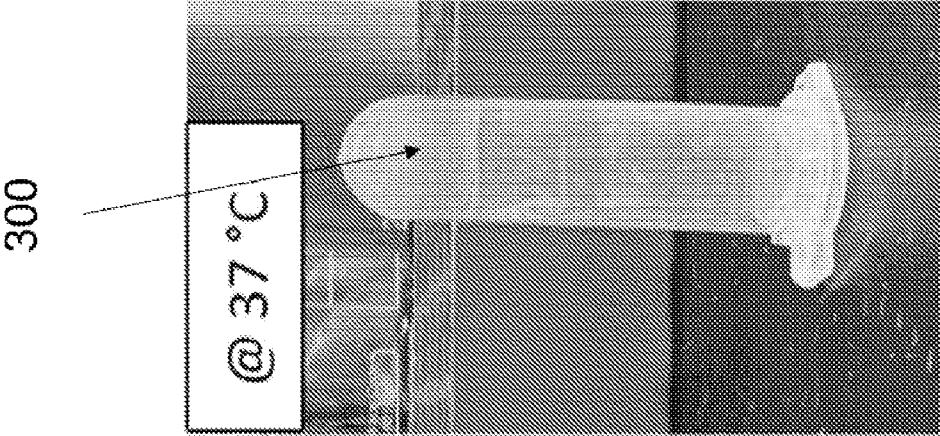


Figure 3B

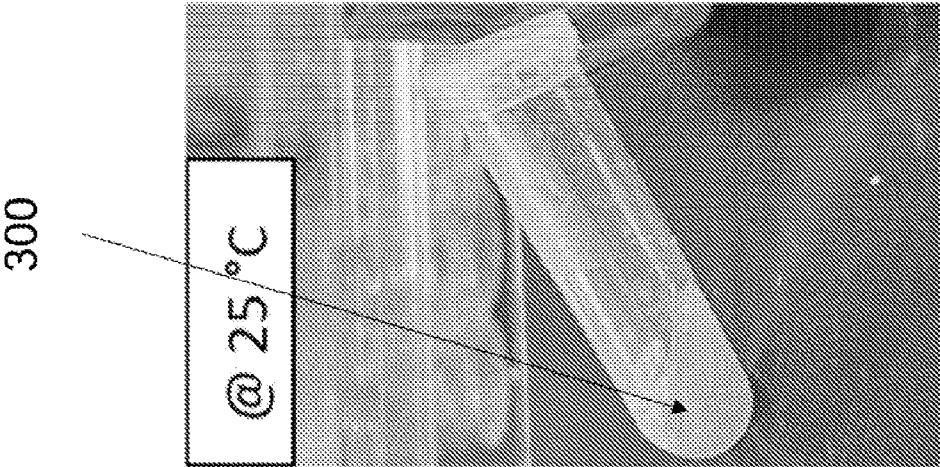
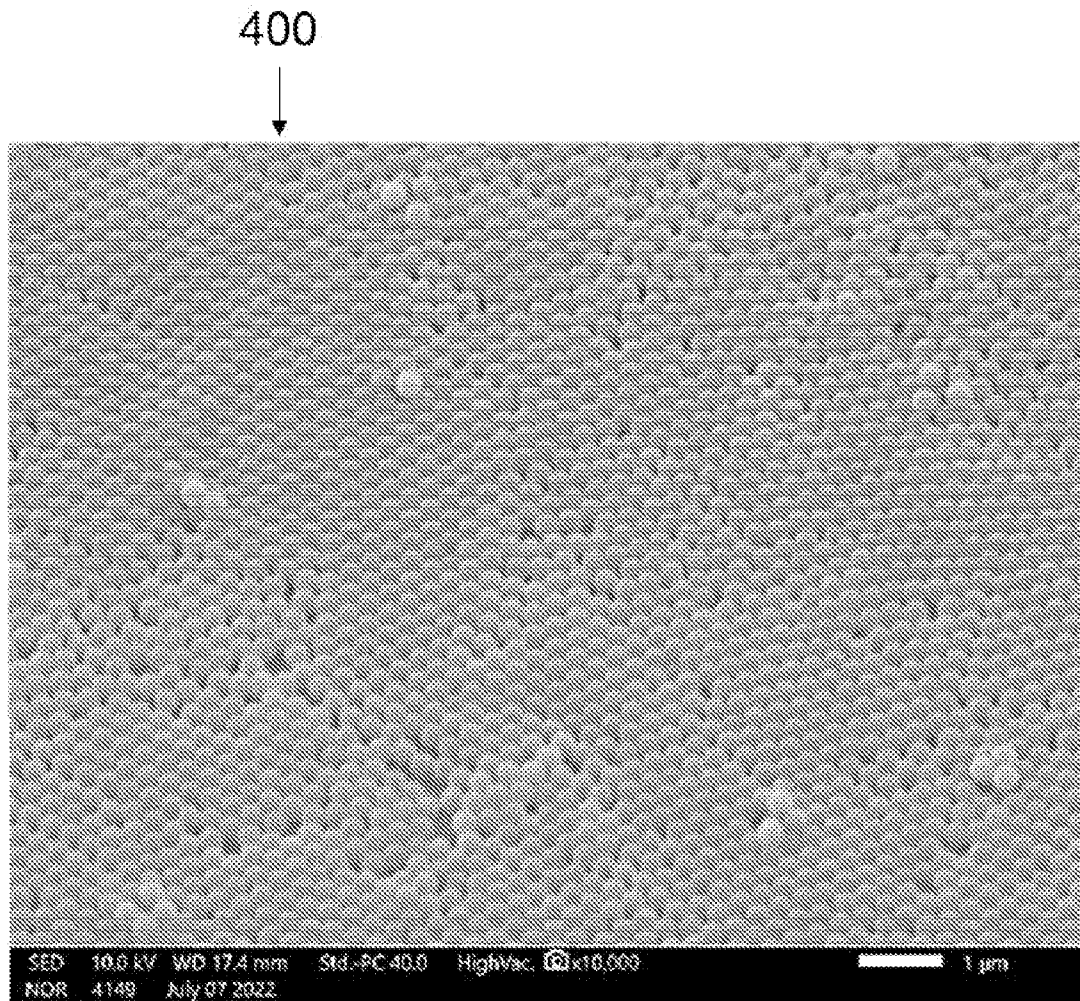


Figure 3A

**Figure 4**

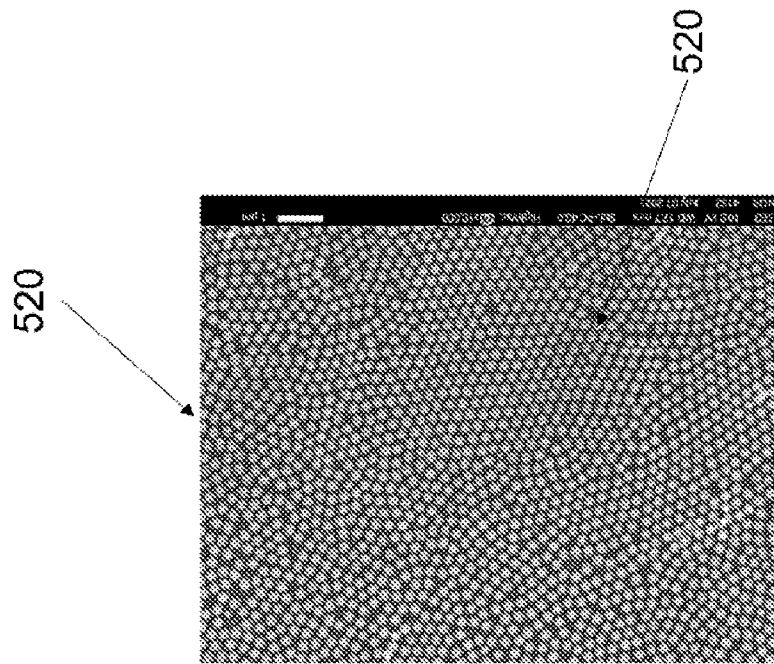


Figure 5C

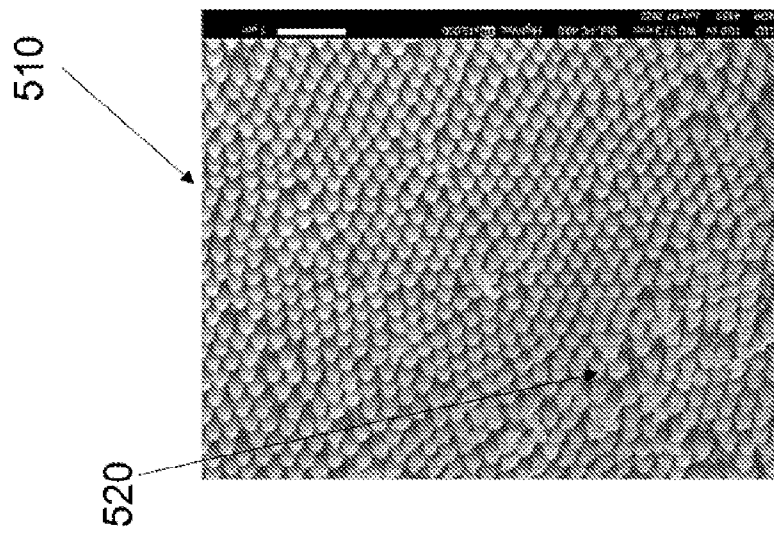


Figure 5B

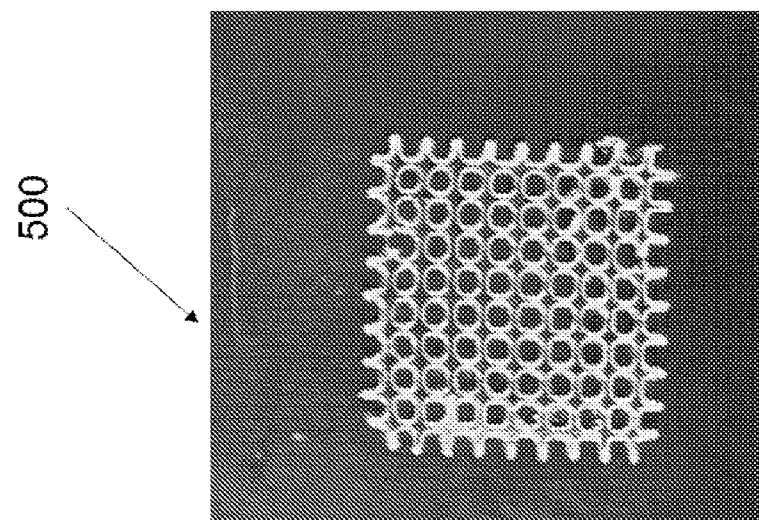


Figure 5A

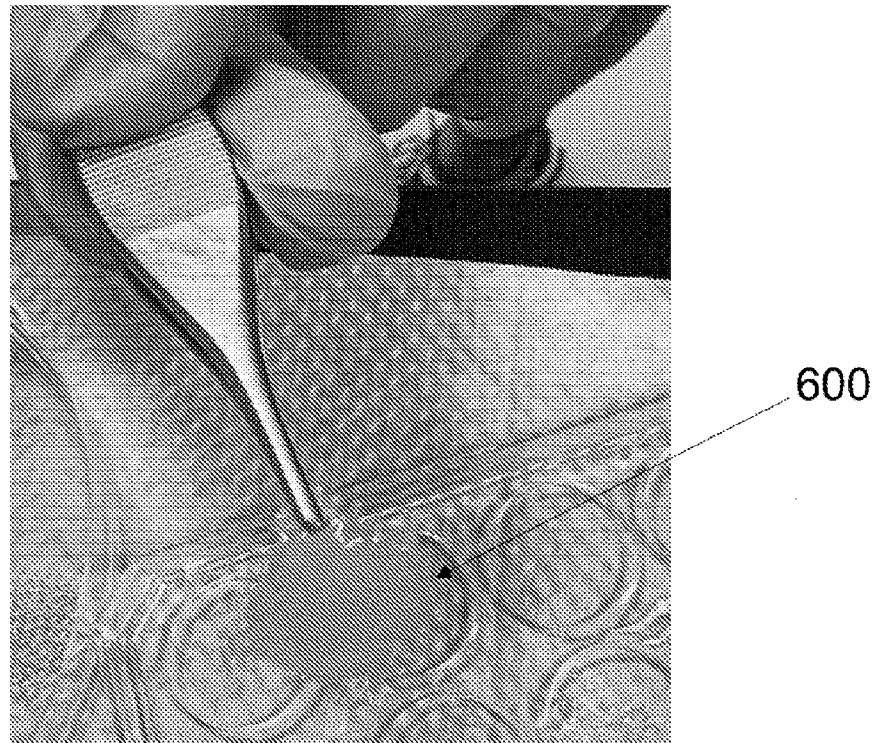


Figure 6

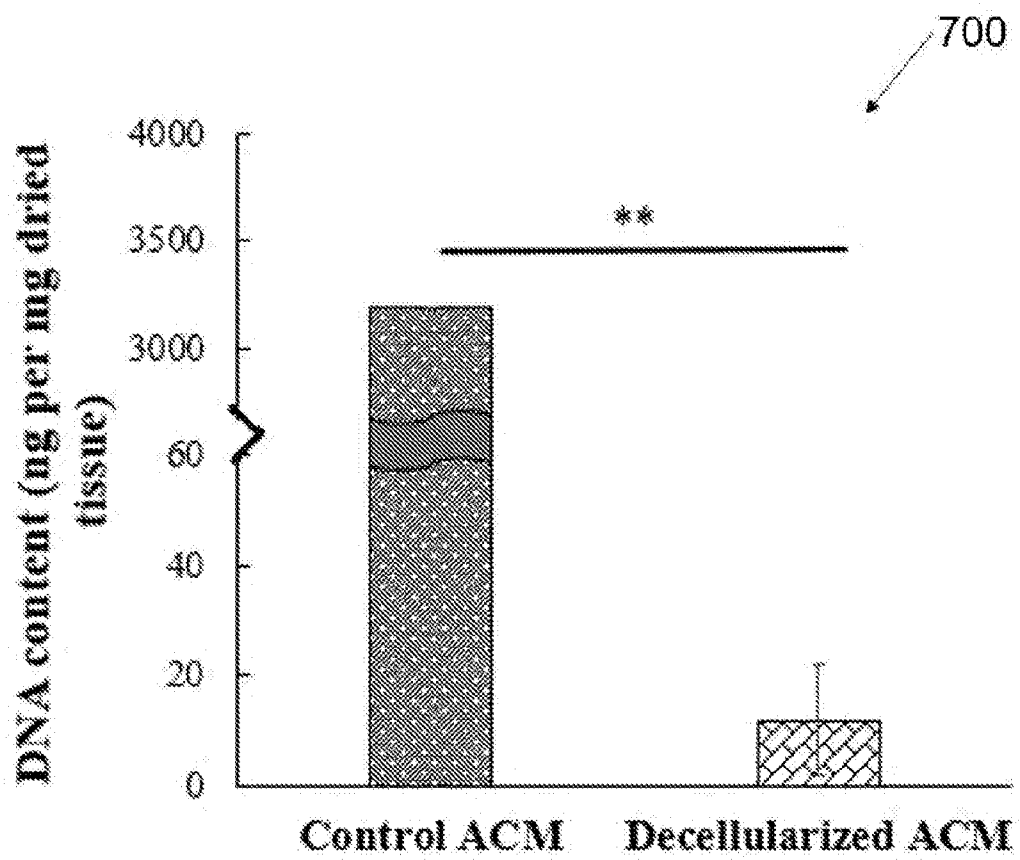


Figure 7

810

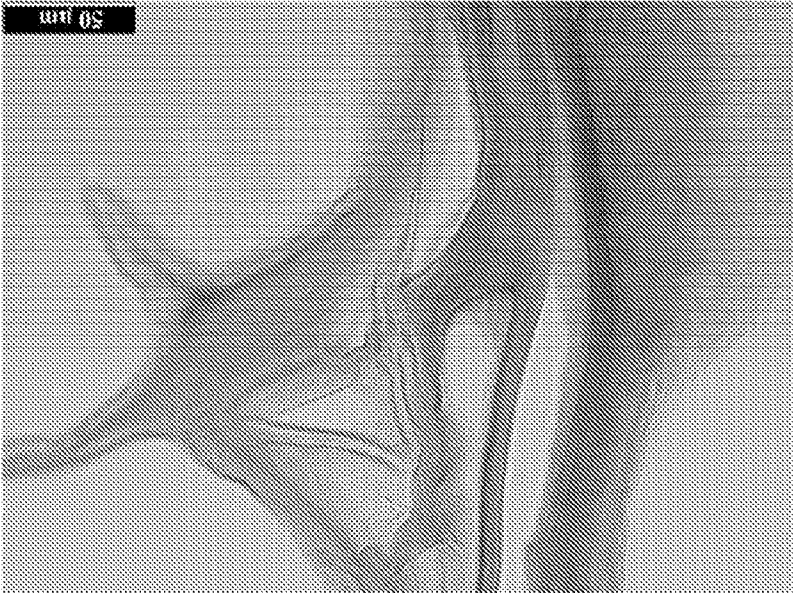


Figure 8B

800

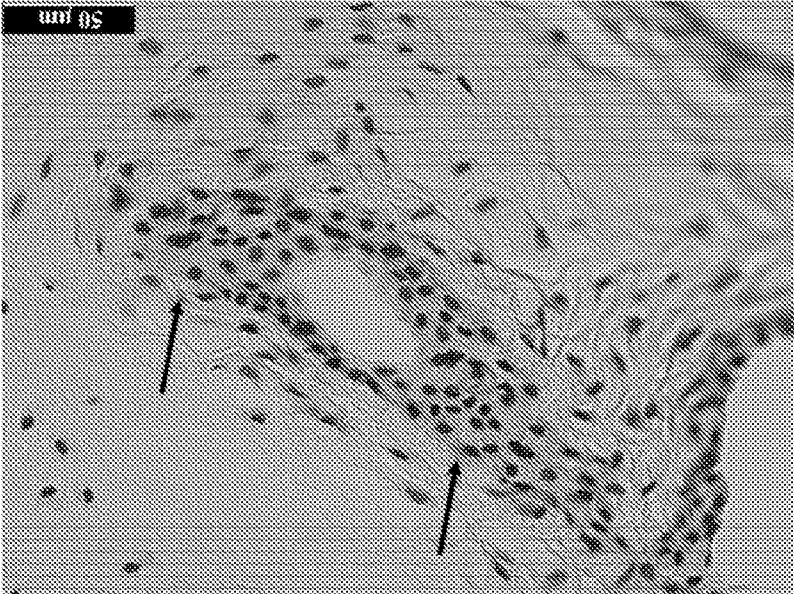


Figure 8A

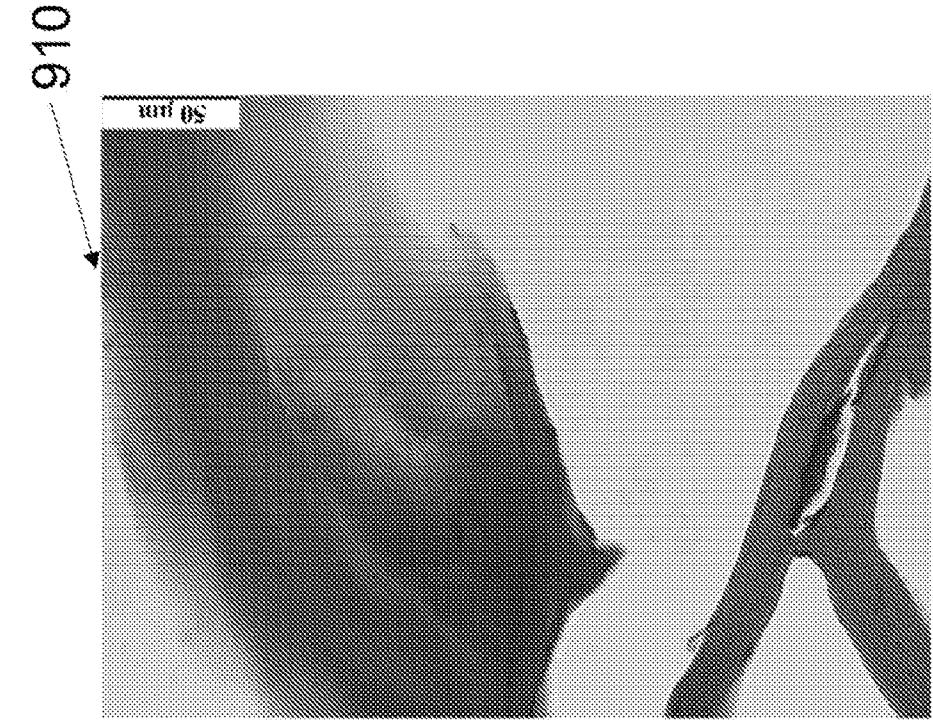


Figure 9B

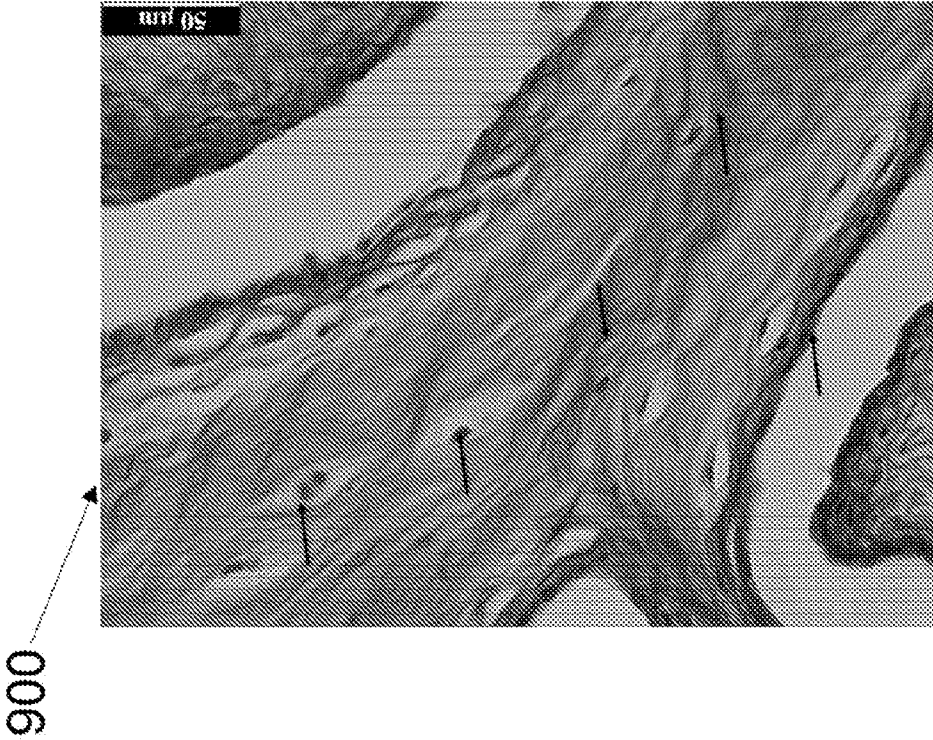


Figure 9A

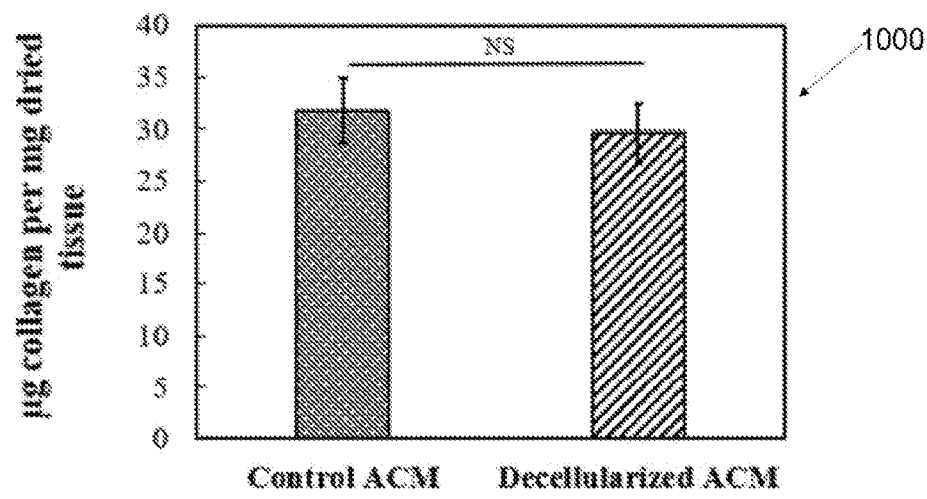


Figure 10

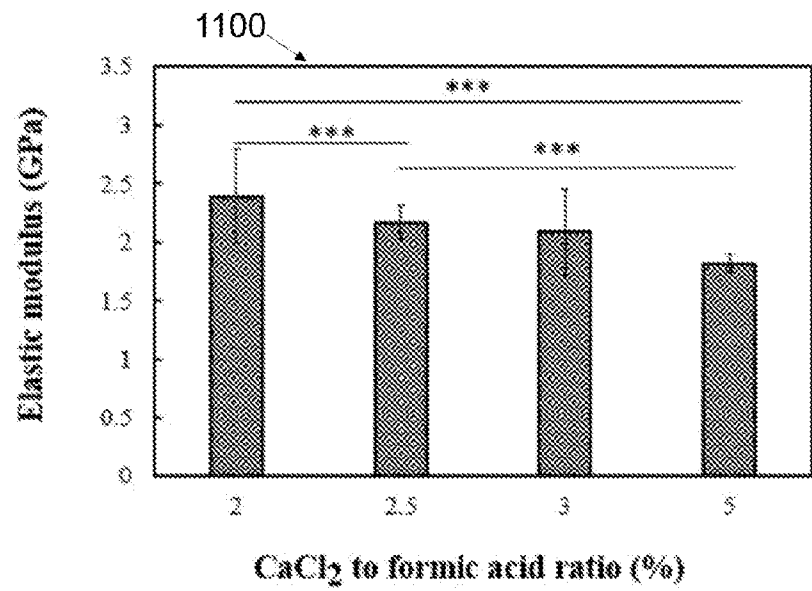


Figure 11A

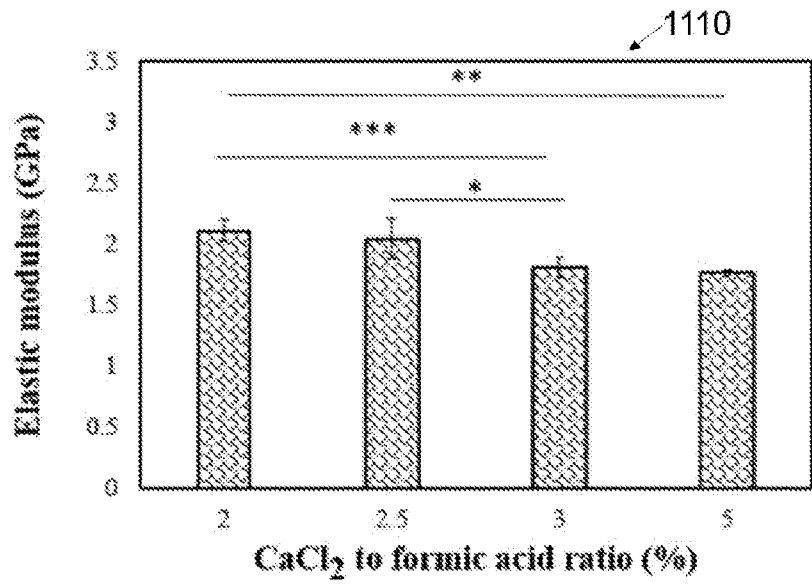


Figure 11B

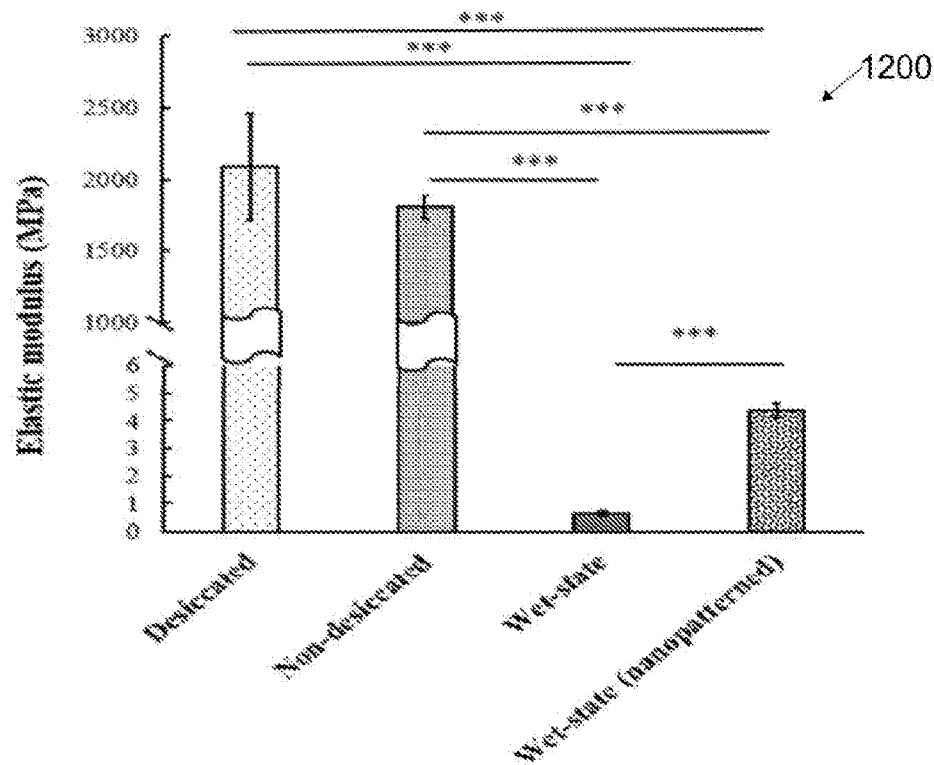


Figure 12

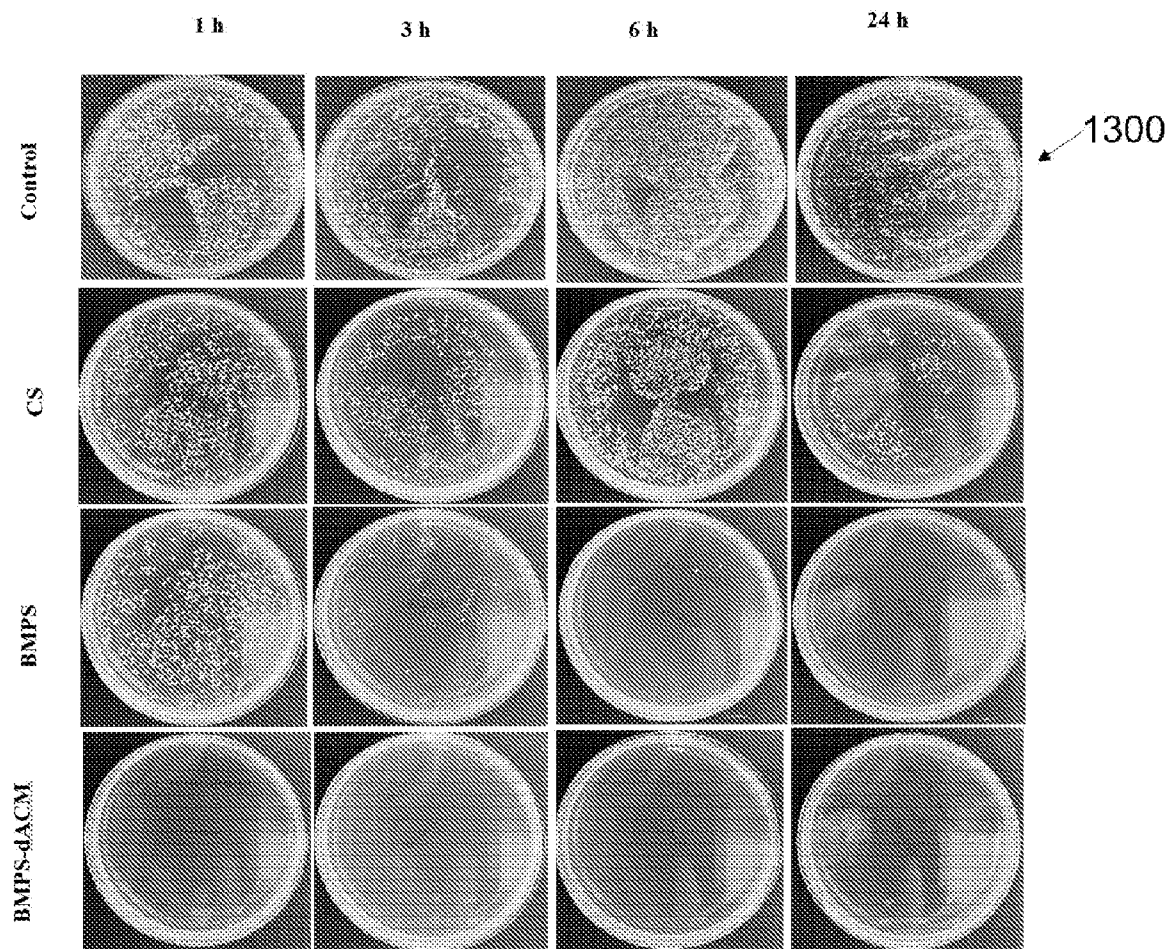


Figure 13

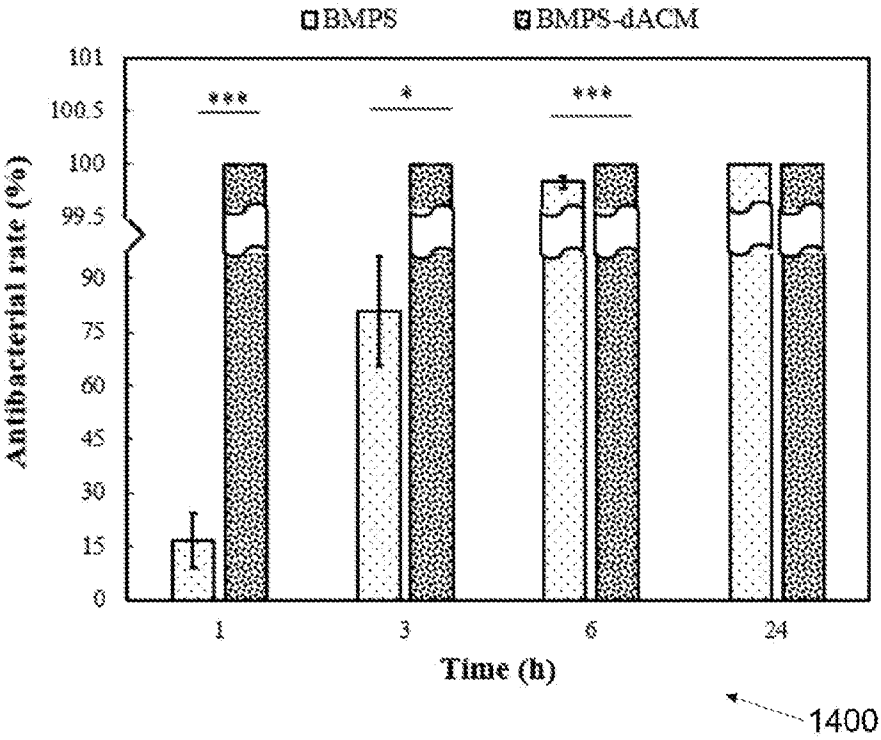


Figure 14

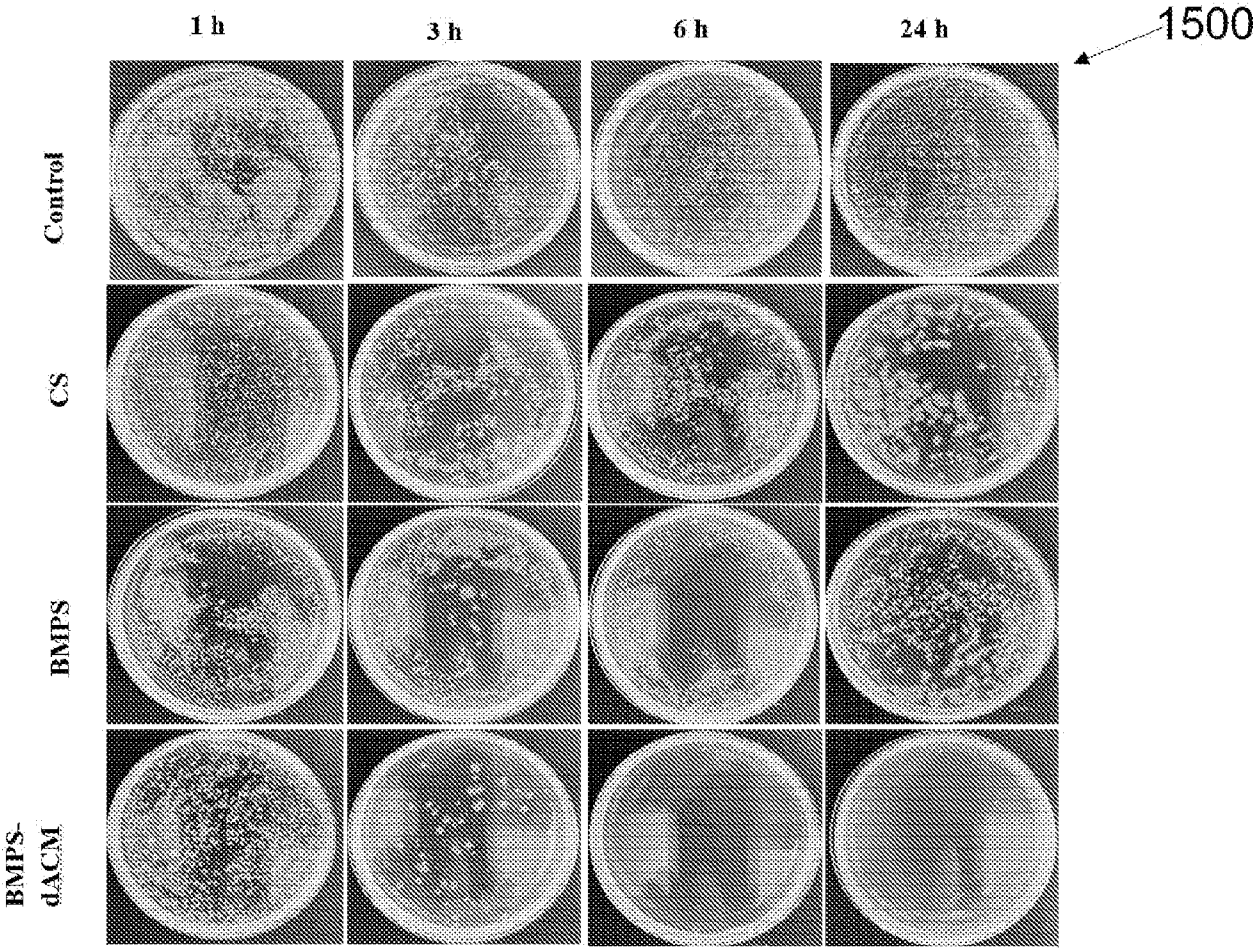


Figure 15

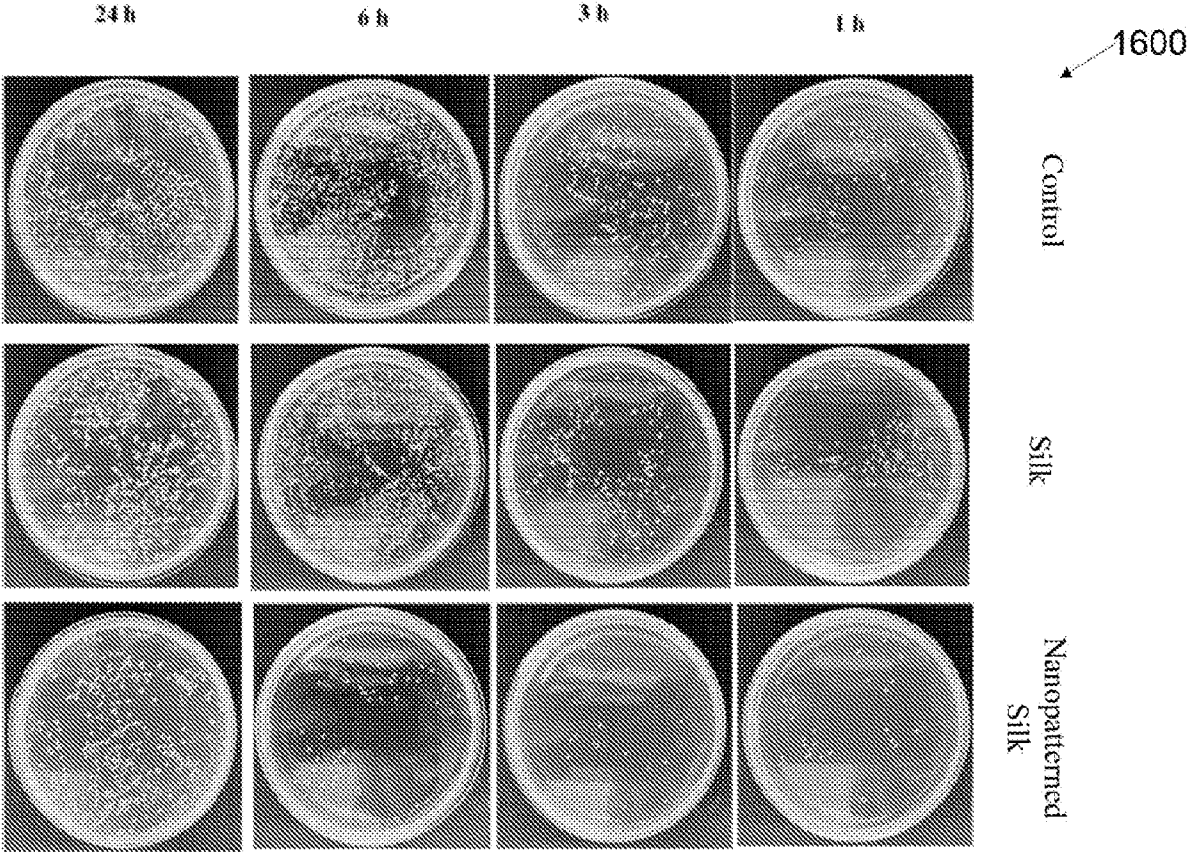


Figure 16

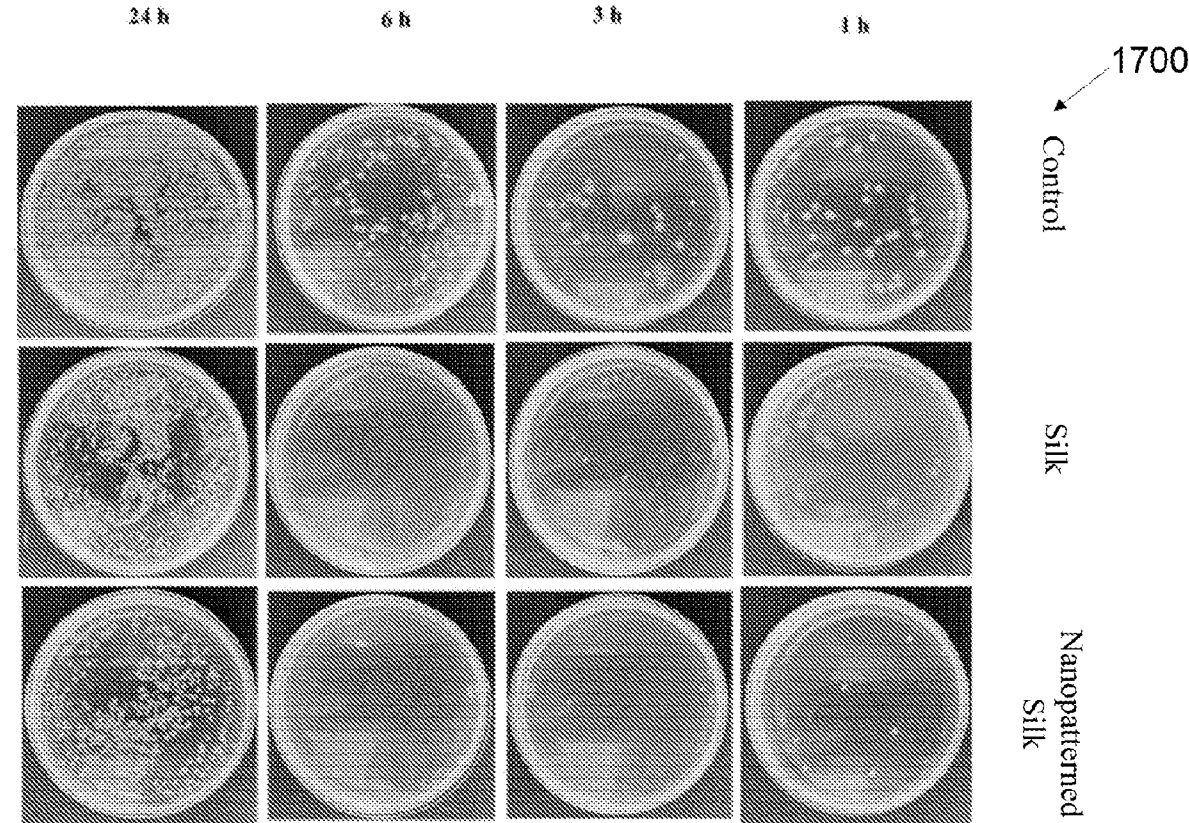


Figure 17

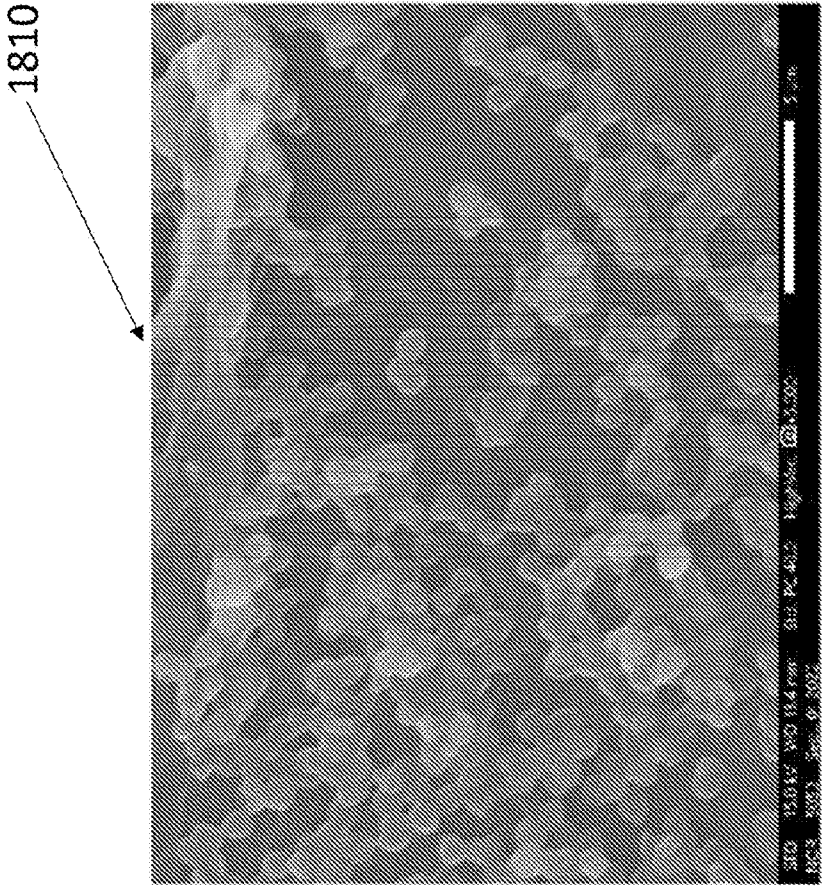


Figure 18B

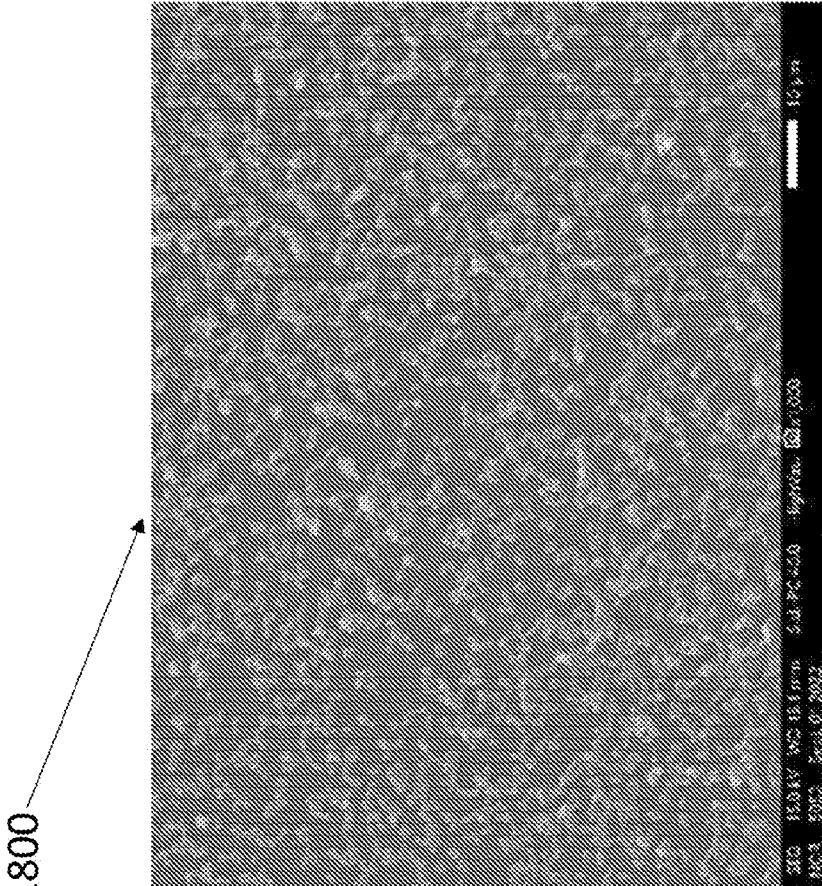


Figure 18A

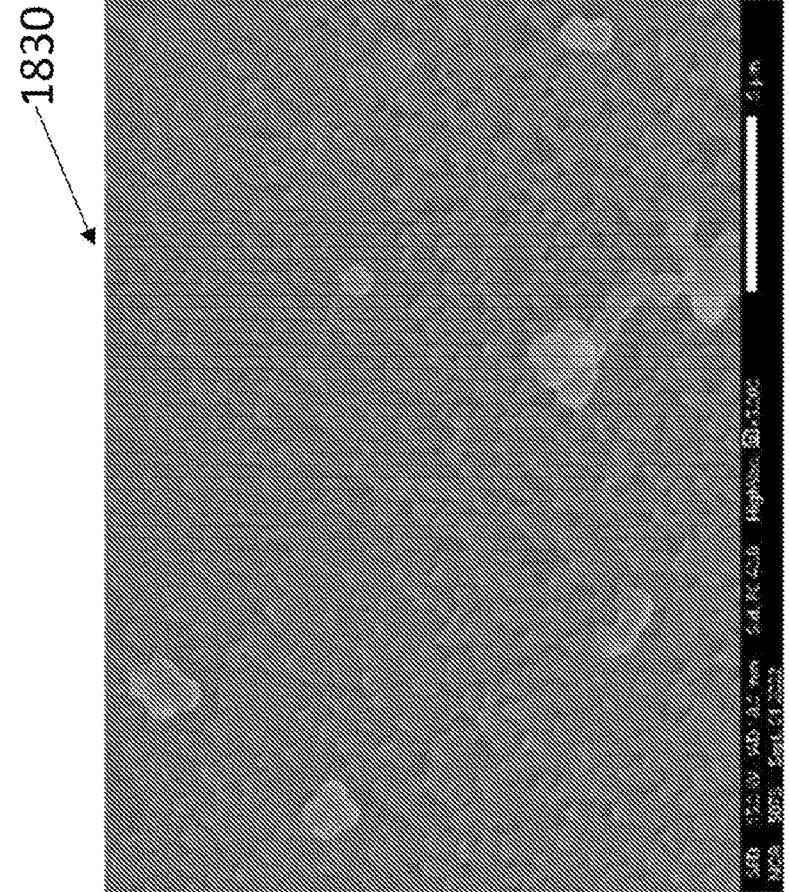


Figure 18D

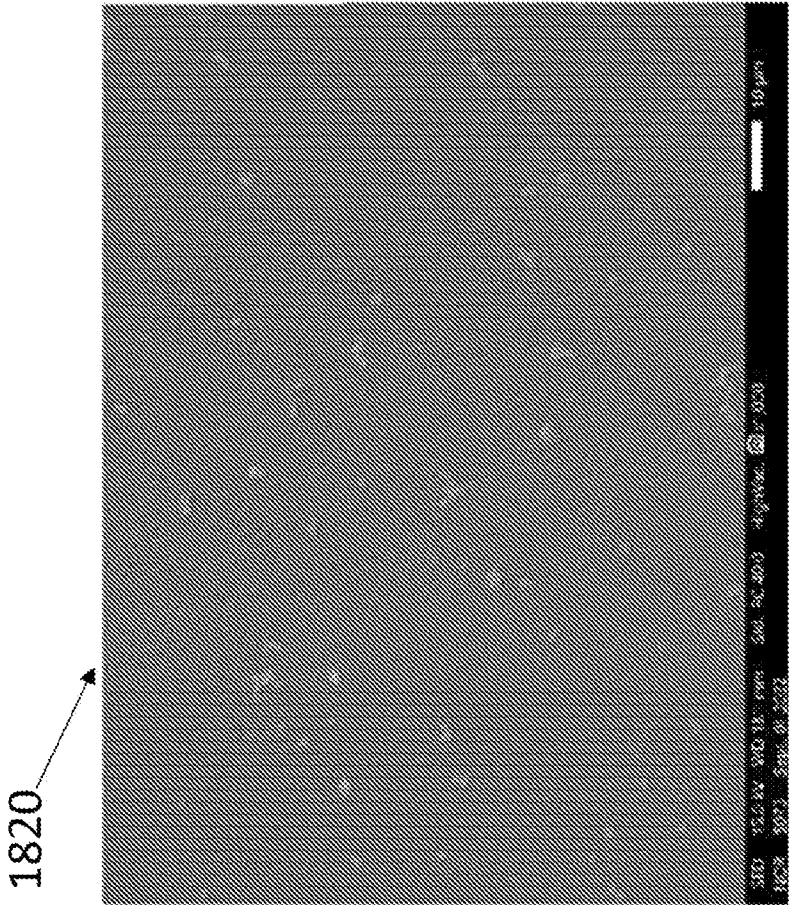


Figure 18C

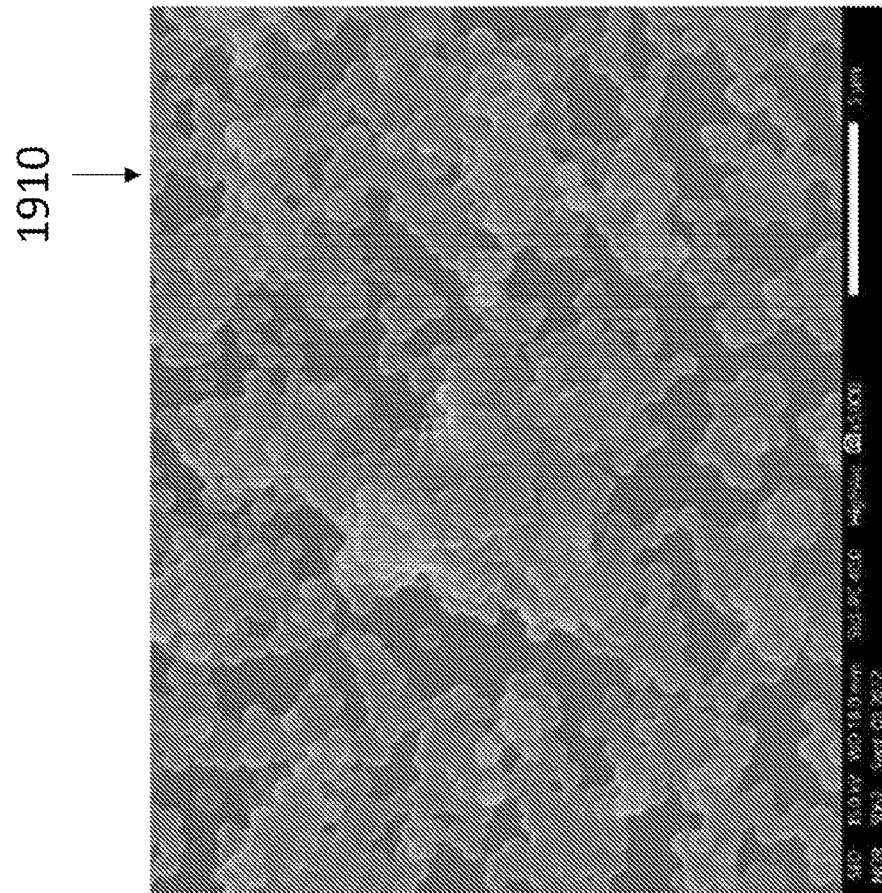


Figure 19B

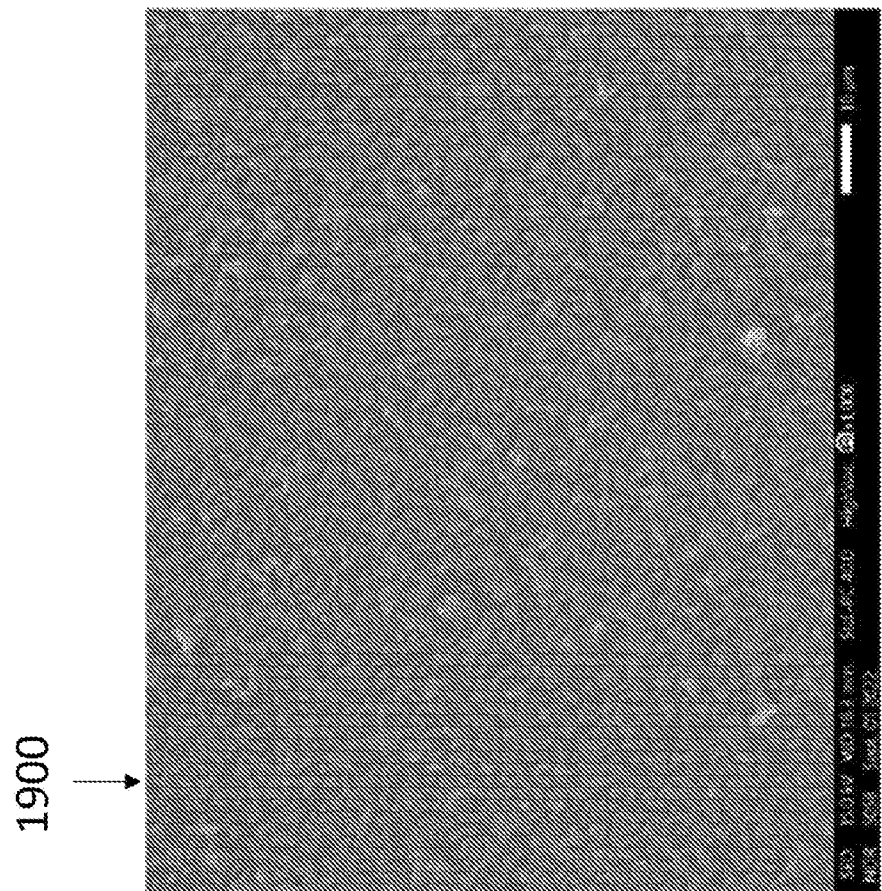


Figure 19A

1930

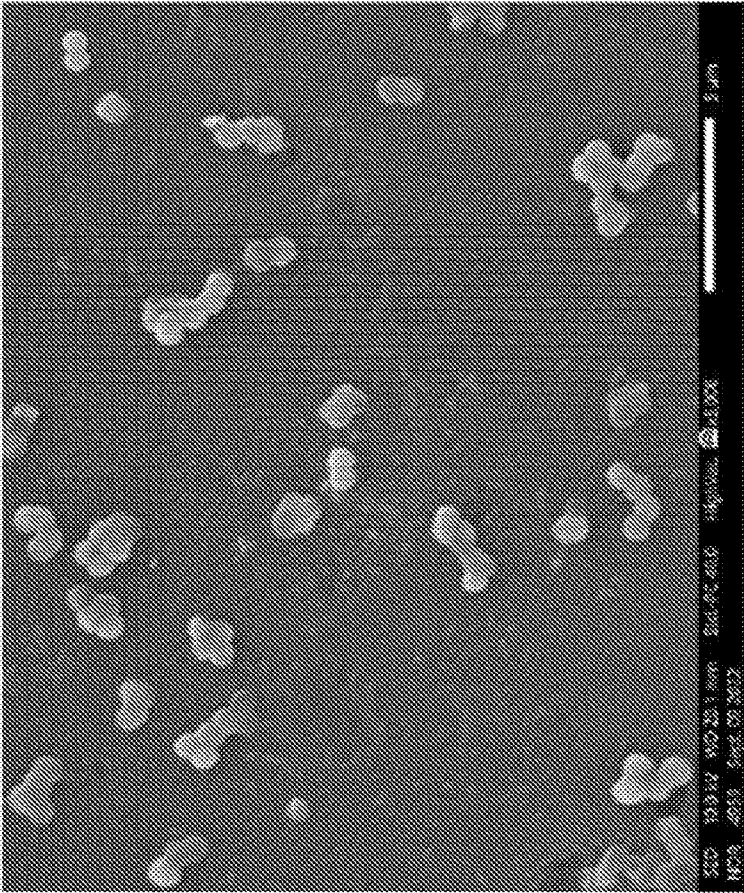


Figure 19D

1920

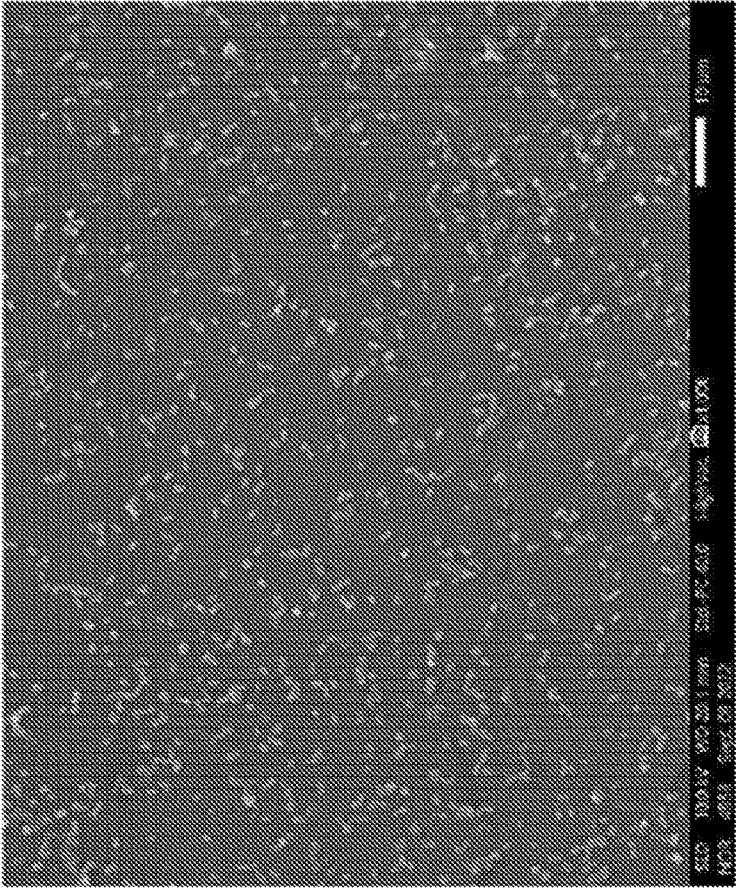


Figure 19C

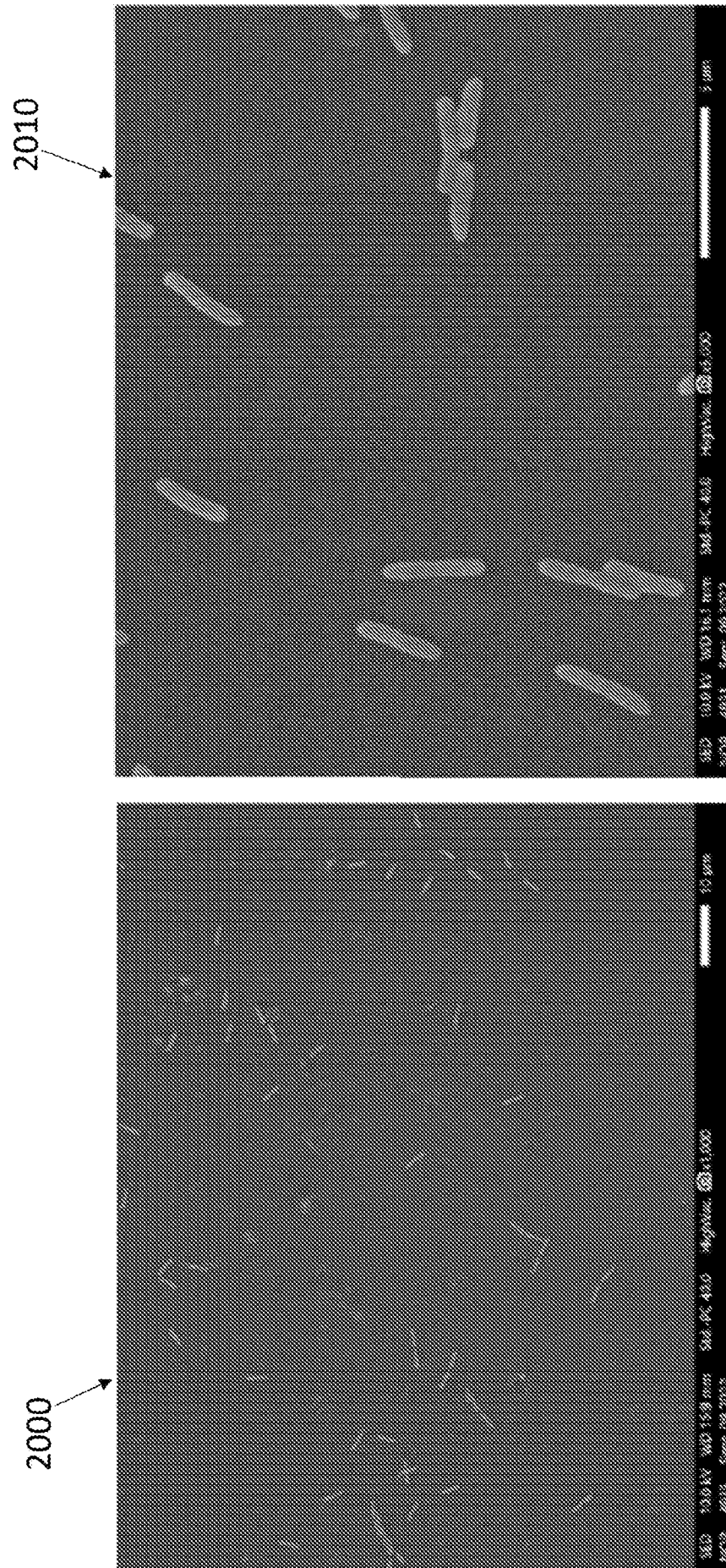


Figure 20B

Figure 20A

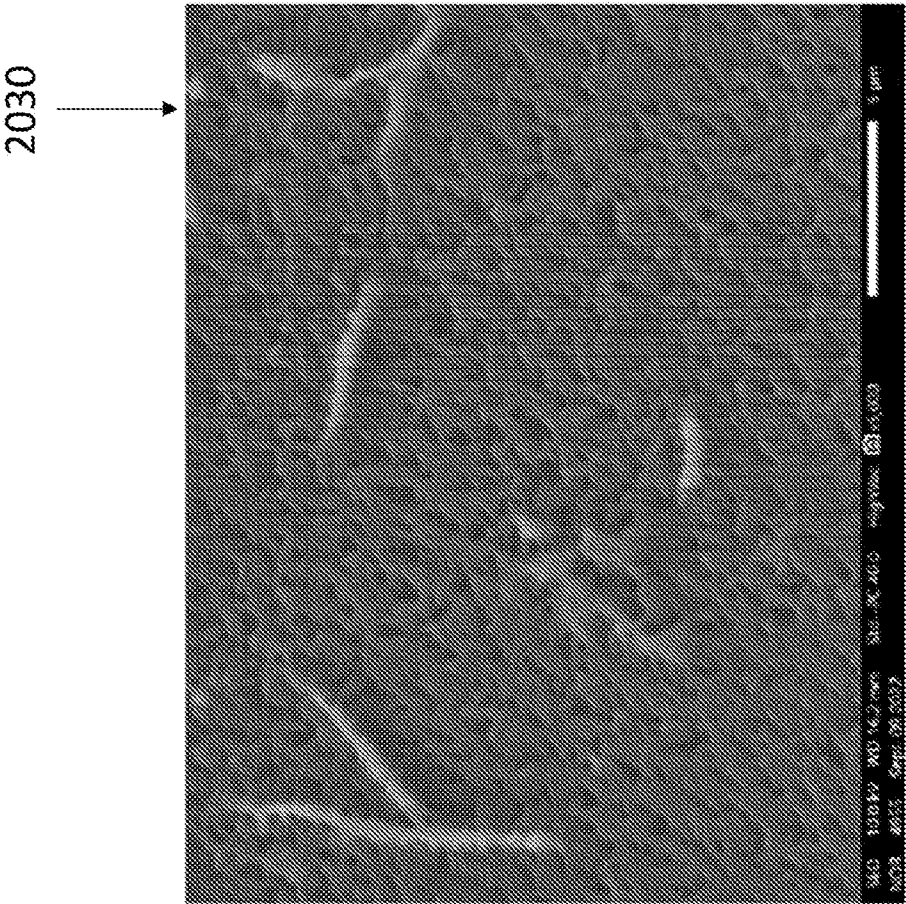


Figure 20D

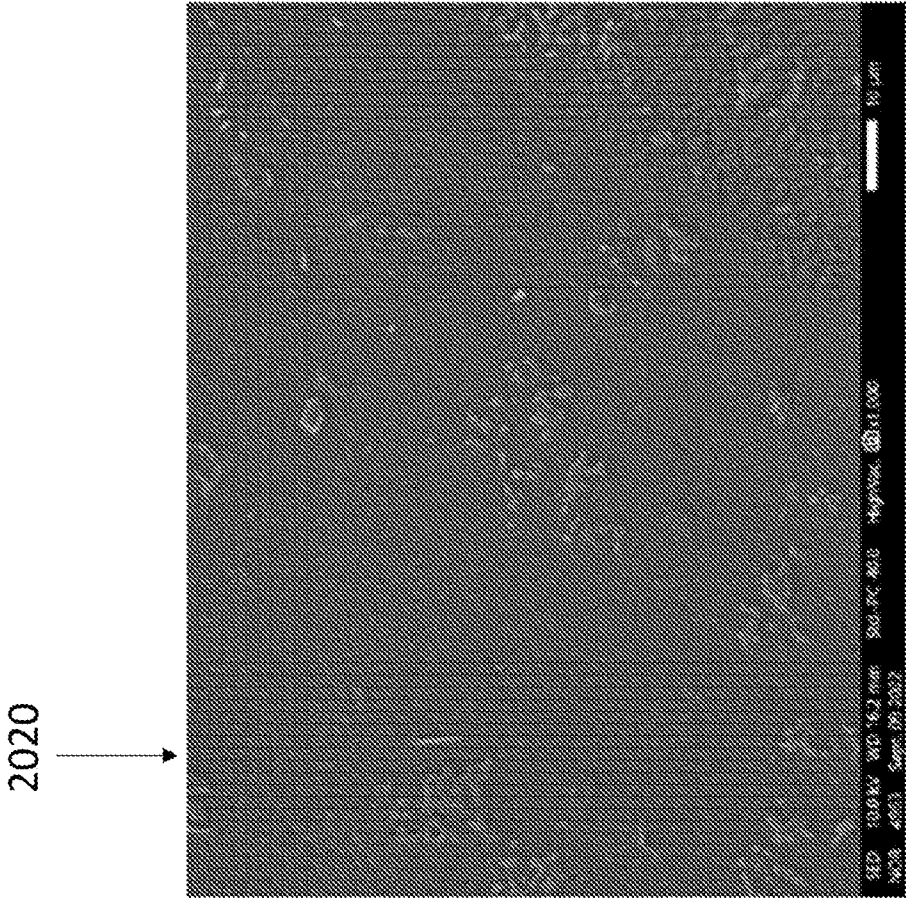


Figure 20C

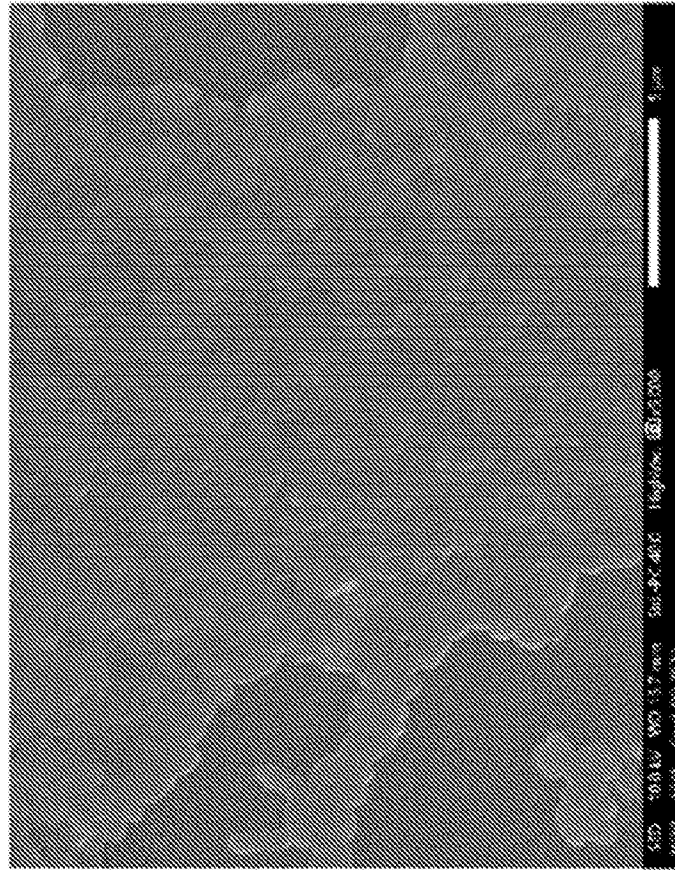
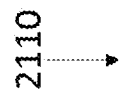


Figure 21B

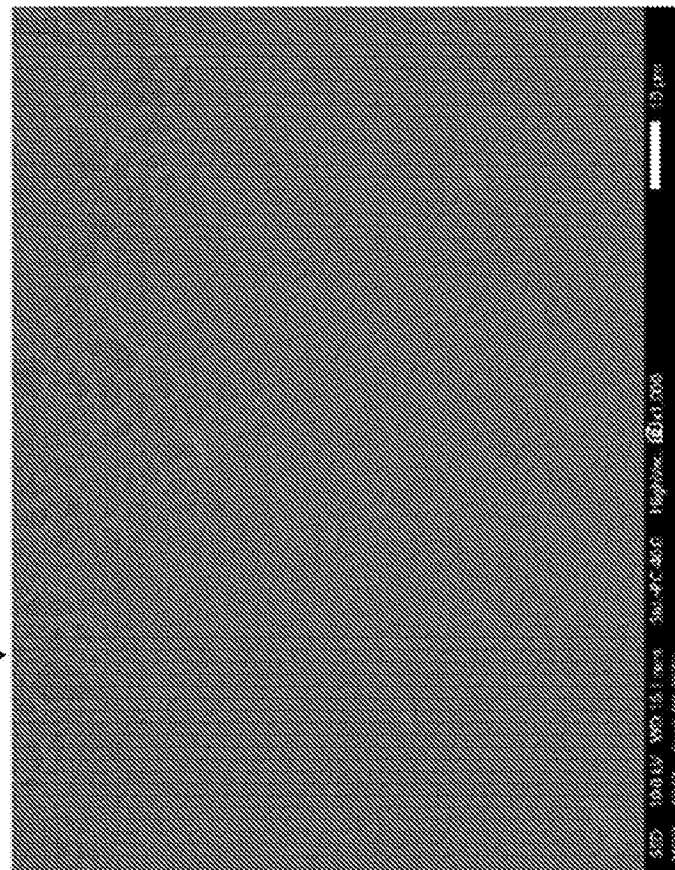
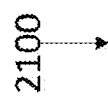


Figure 21A

2130

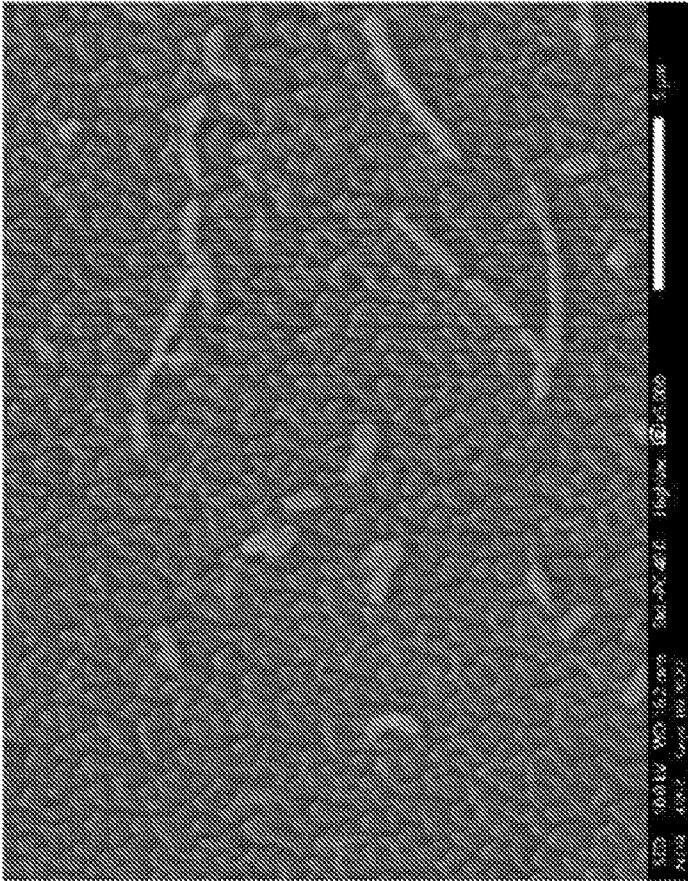


Figure 21D

2120

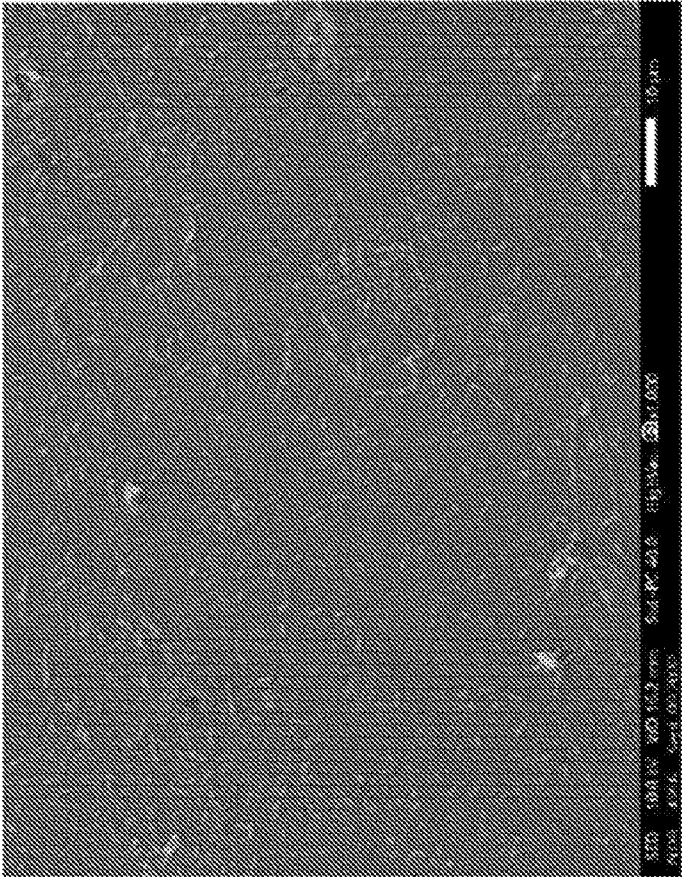


Figure 21C

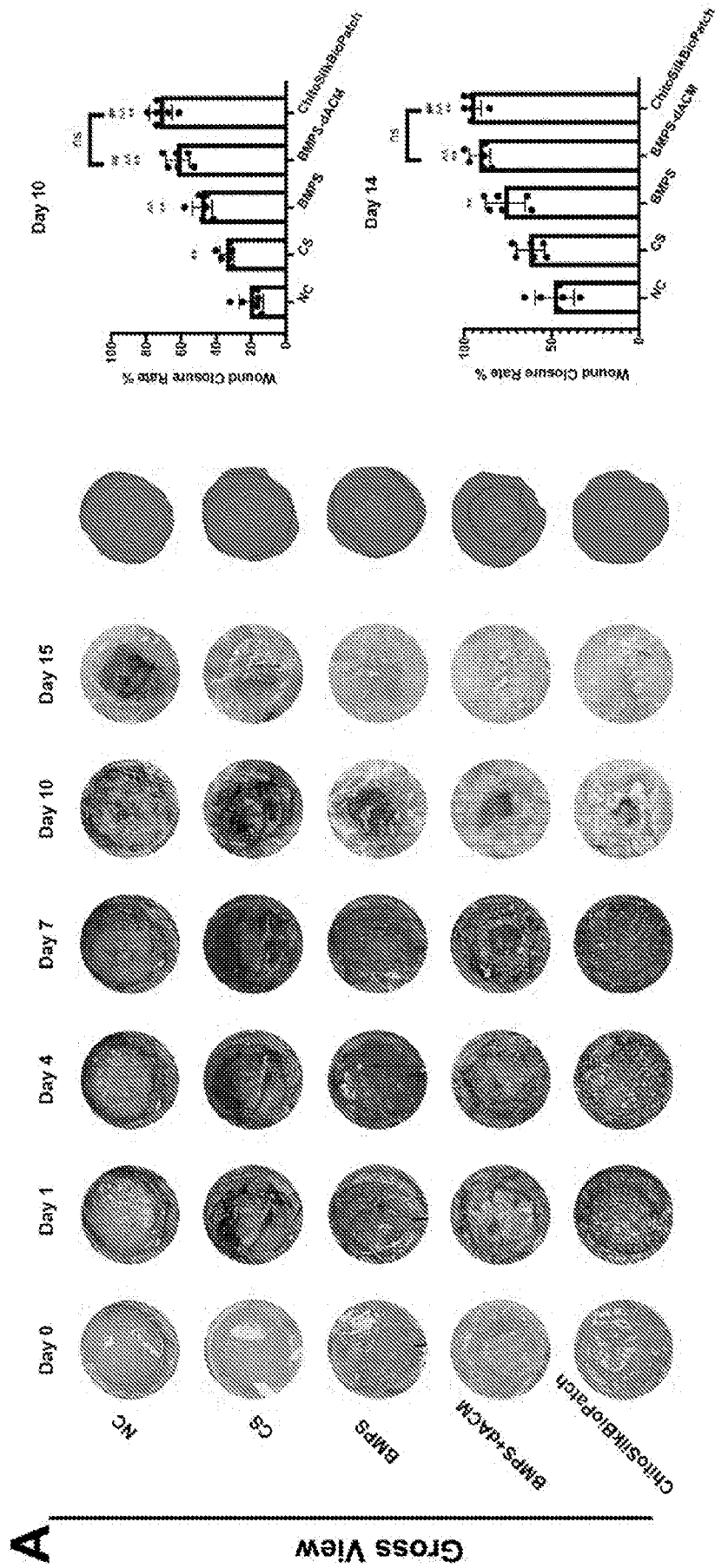


Figure 22A

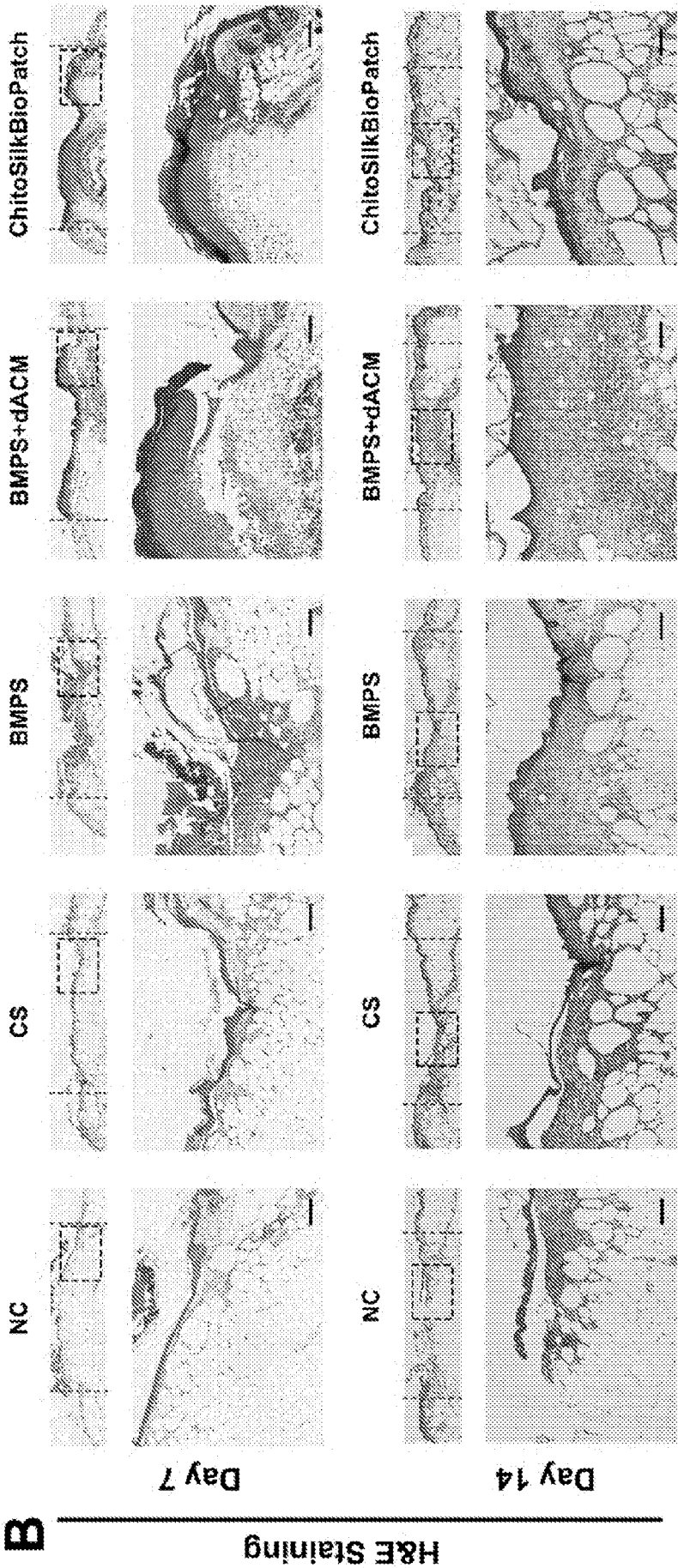


Figure 22B

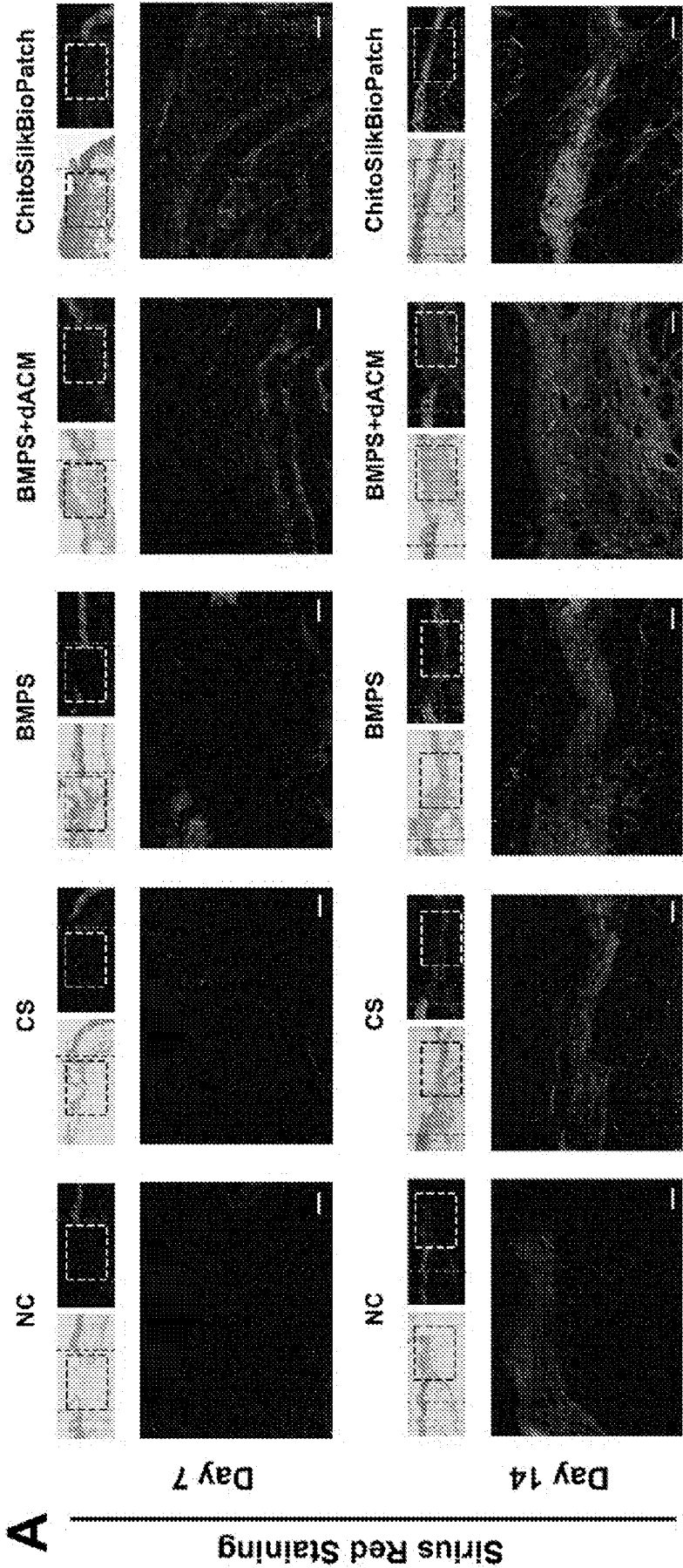


Figure 23A

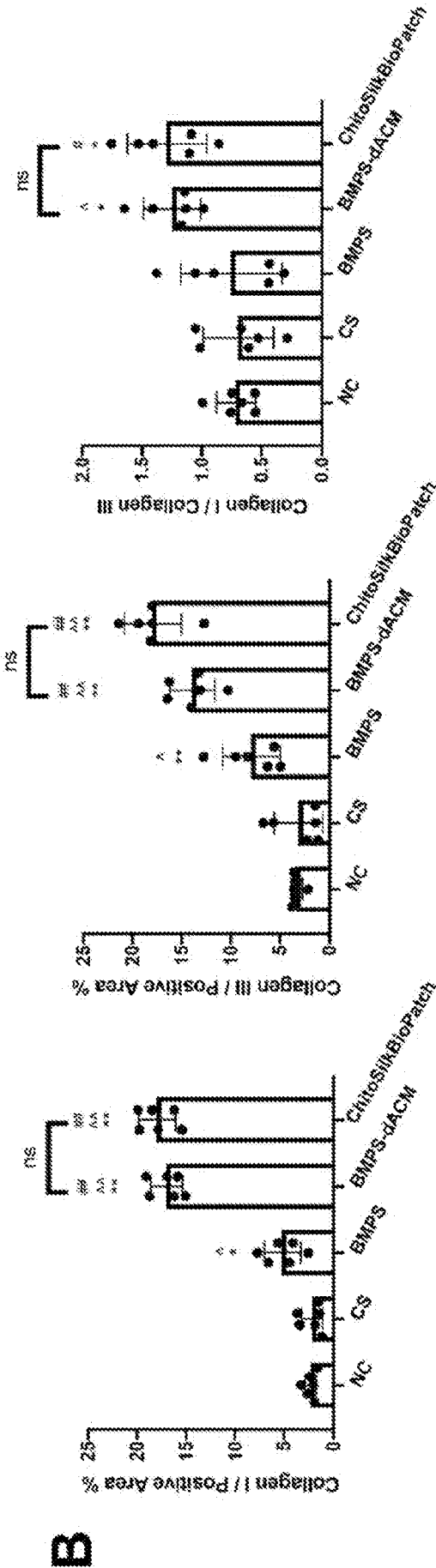


Figure 23B

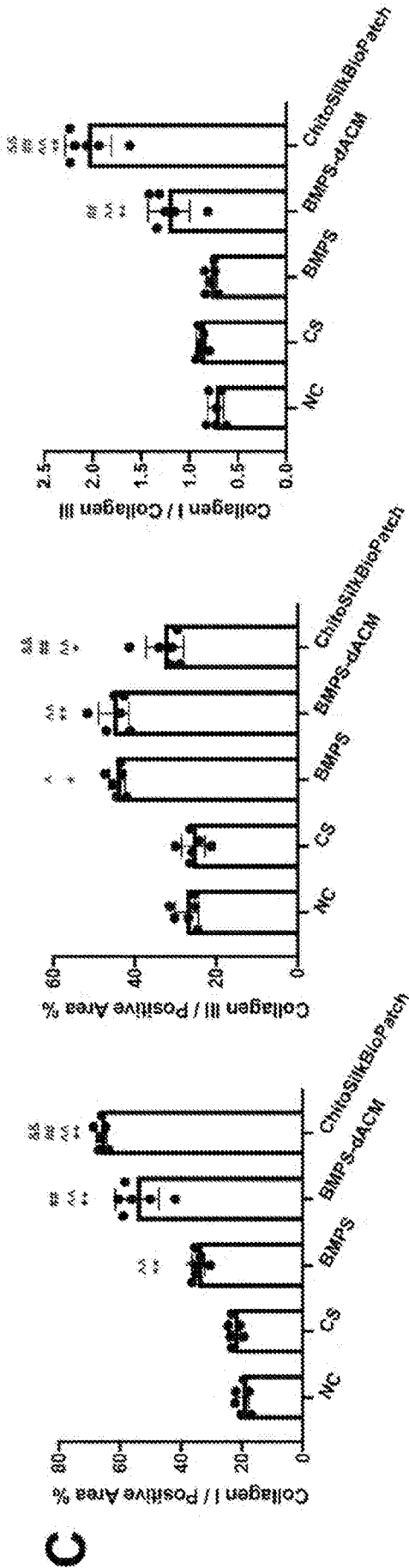


Figure 23C

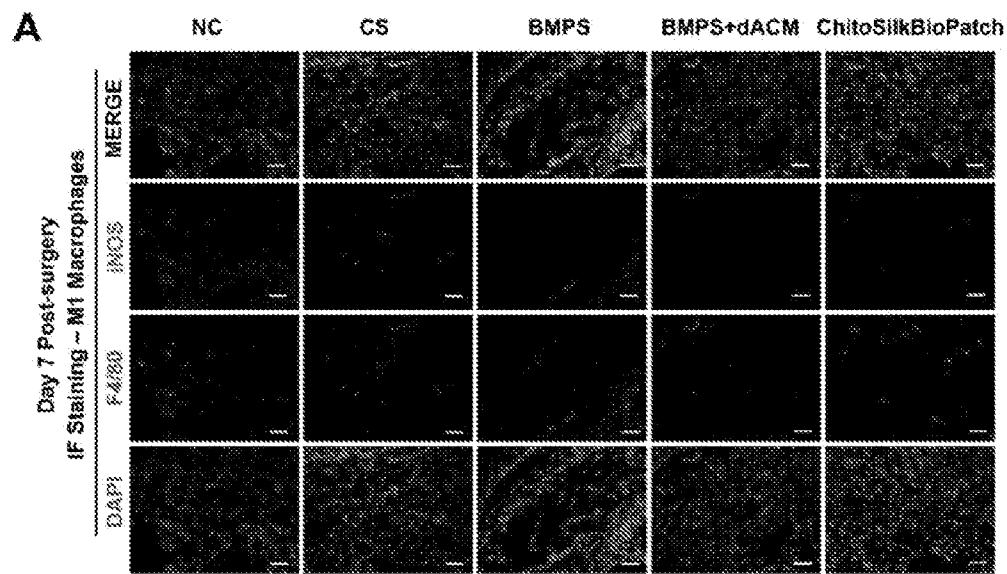


Figure 24A

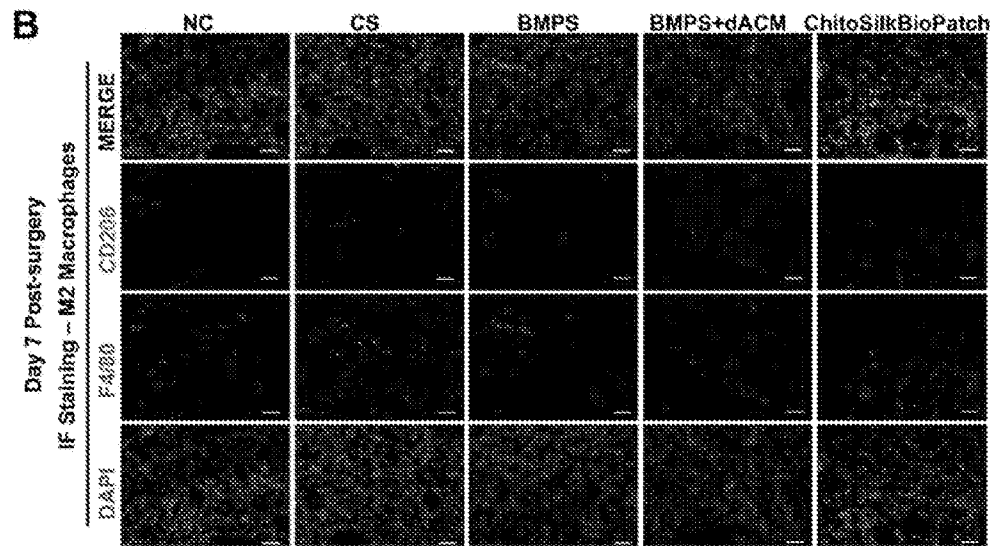


Figure 24B

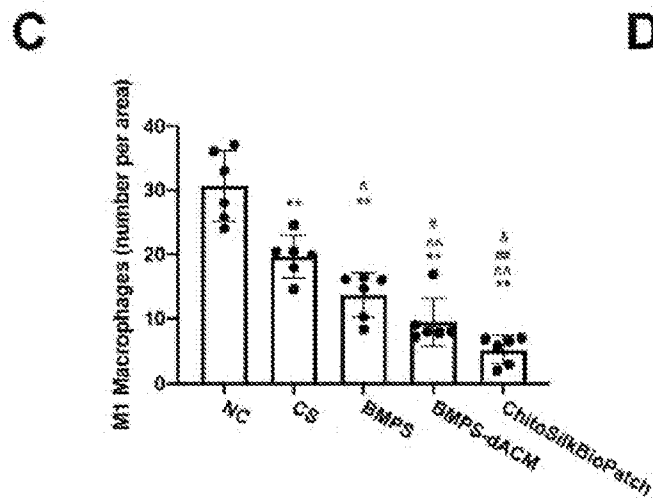


Figure 24C

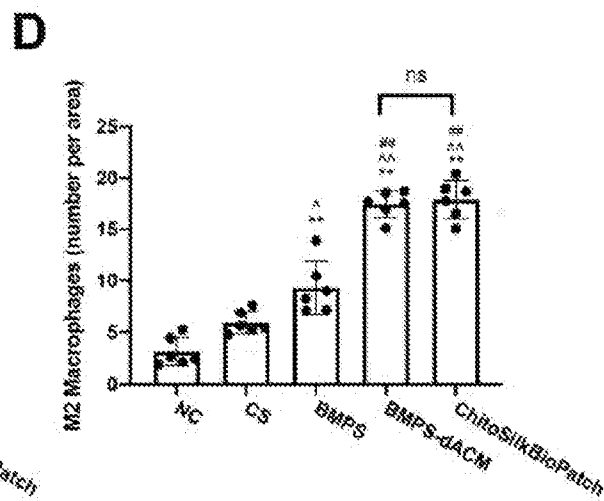


Figure 24D

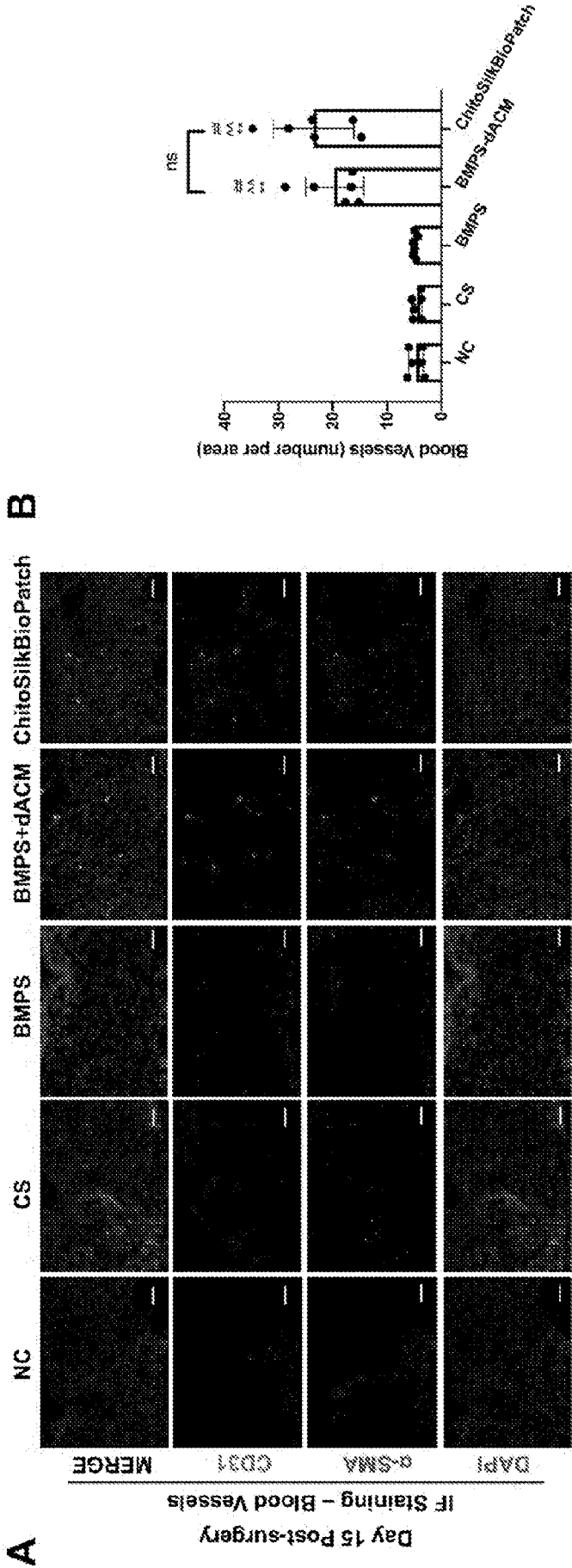


Figure 25A

Figure 25B

2610 →

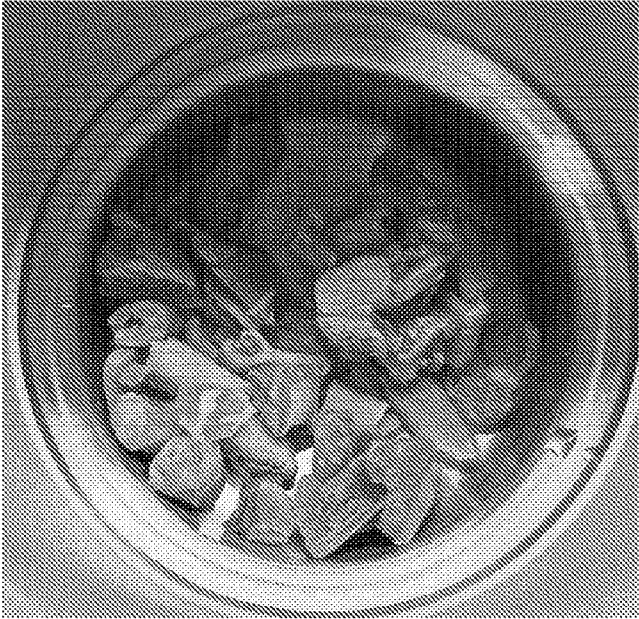


Figure 26B

2600 →

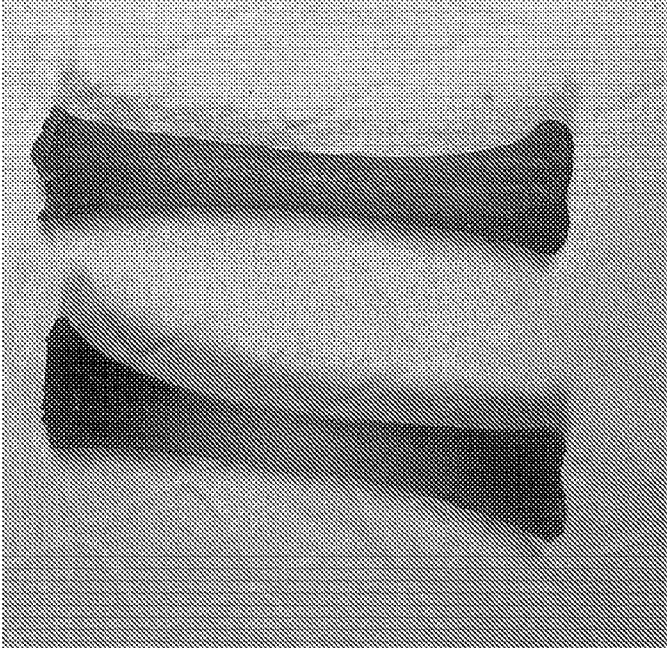


Figure 26A

2630

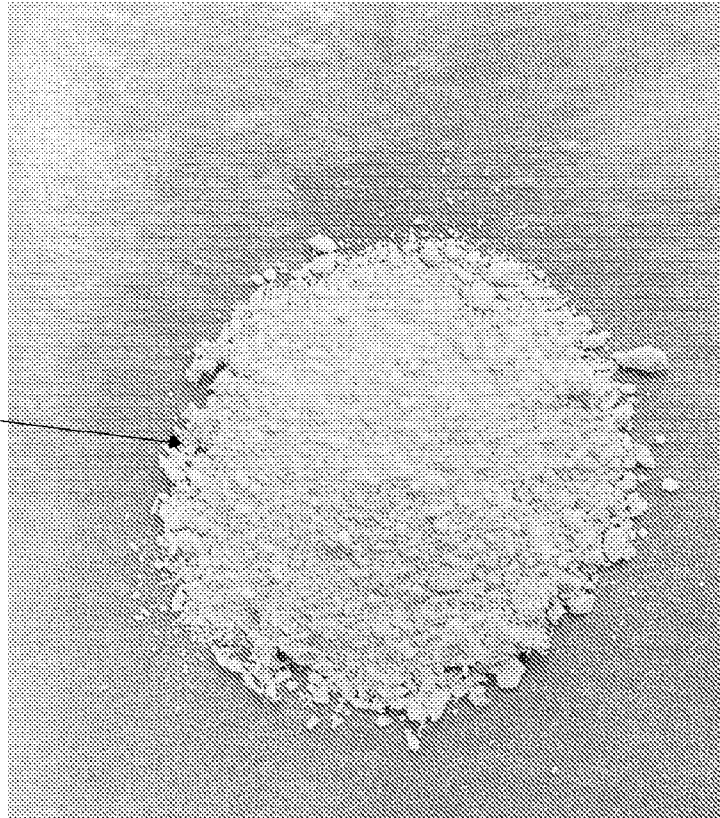


Figure 26D

2620

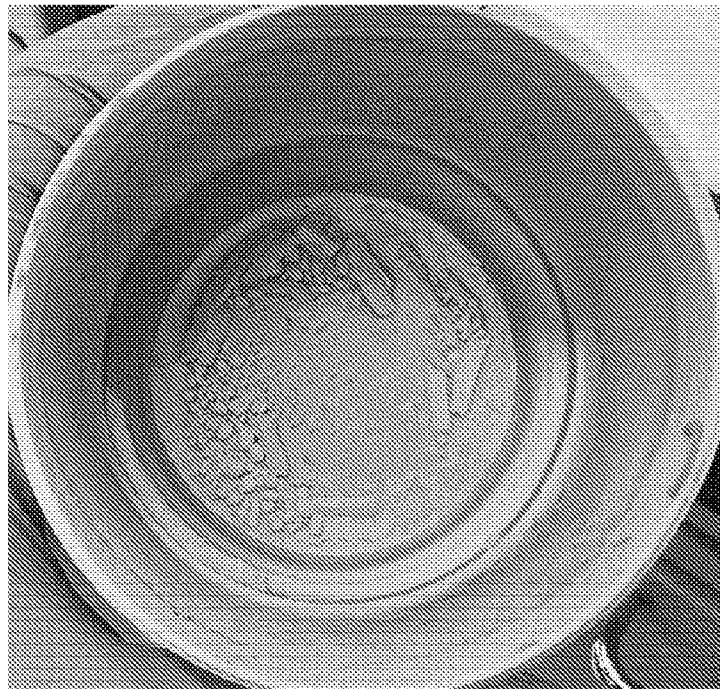


Figure 26C

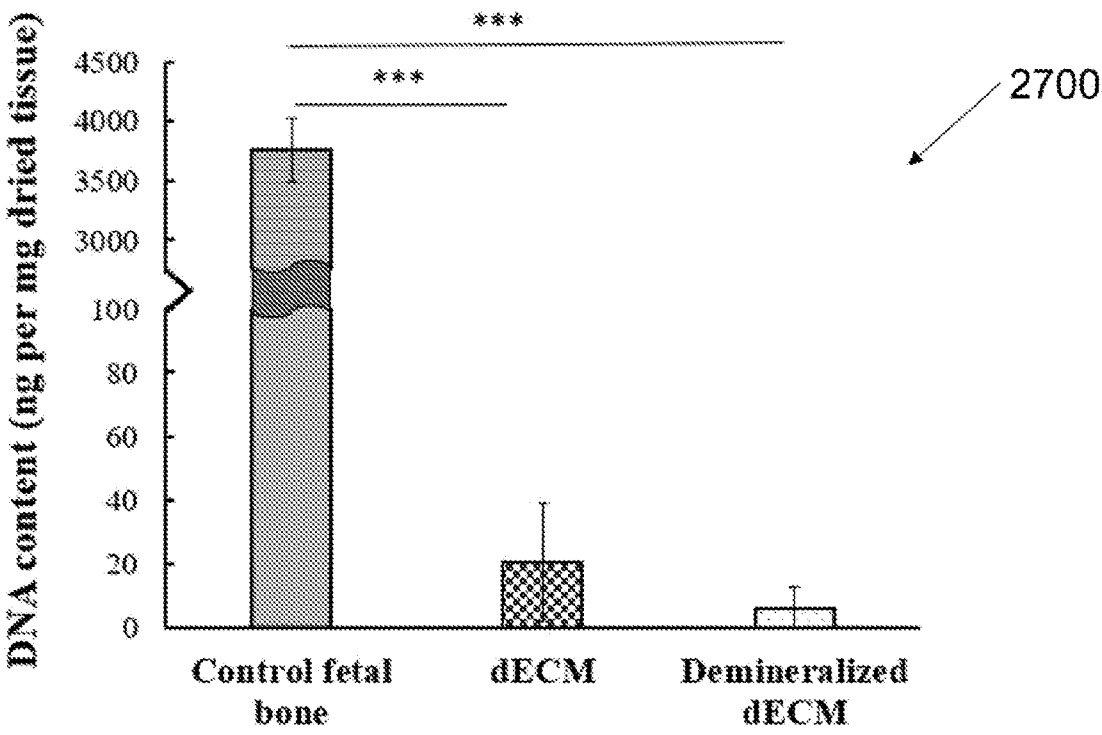


Figure 27

Figure 25A

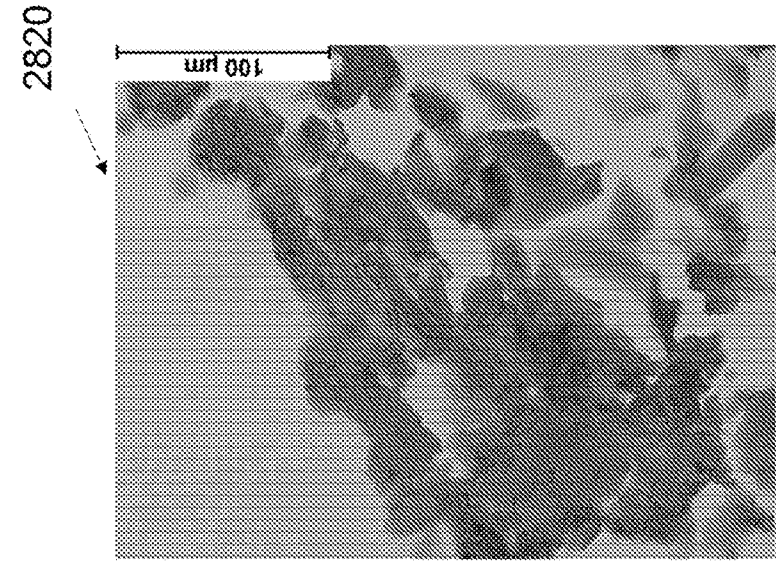


Figure 28C

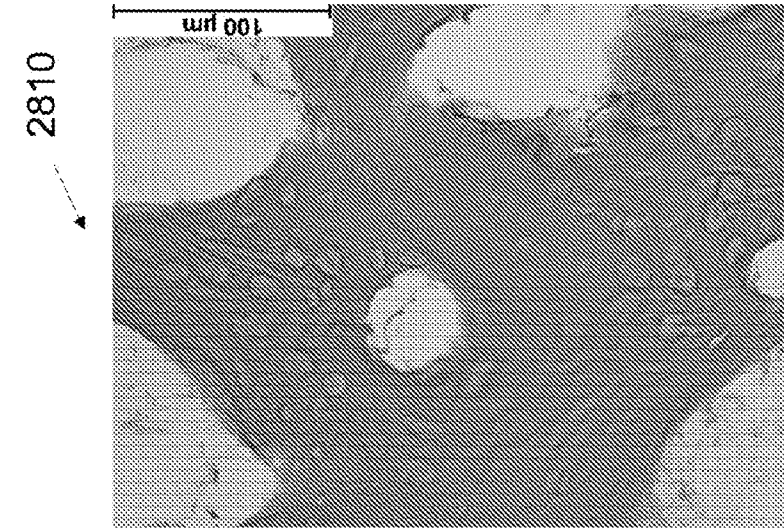


Figure 28B

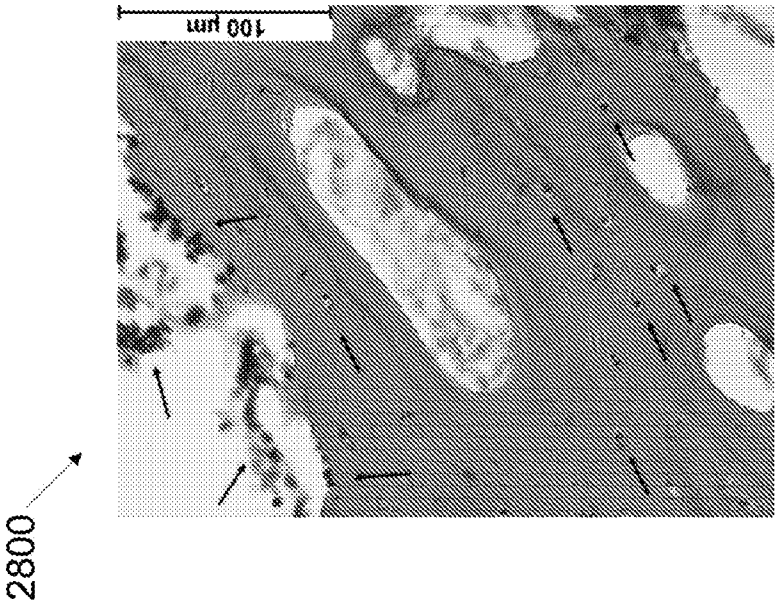


Figure 28A

2920

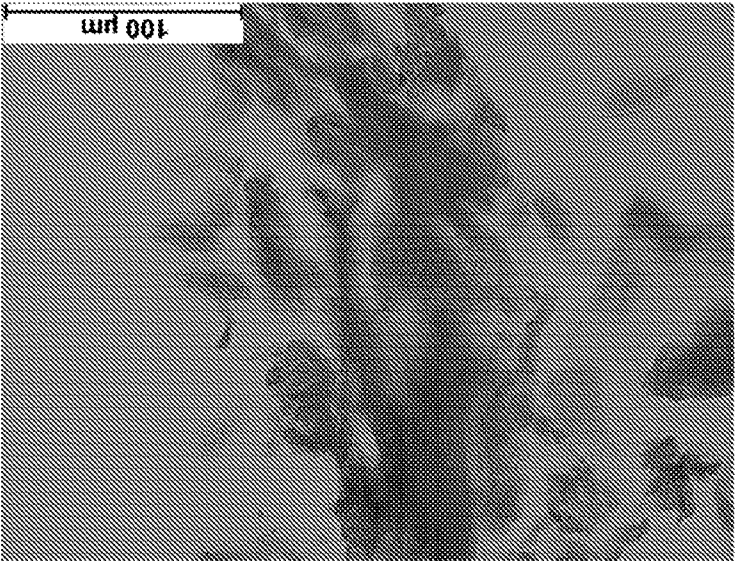


Figure 29C

2910

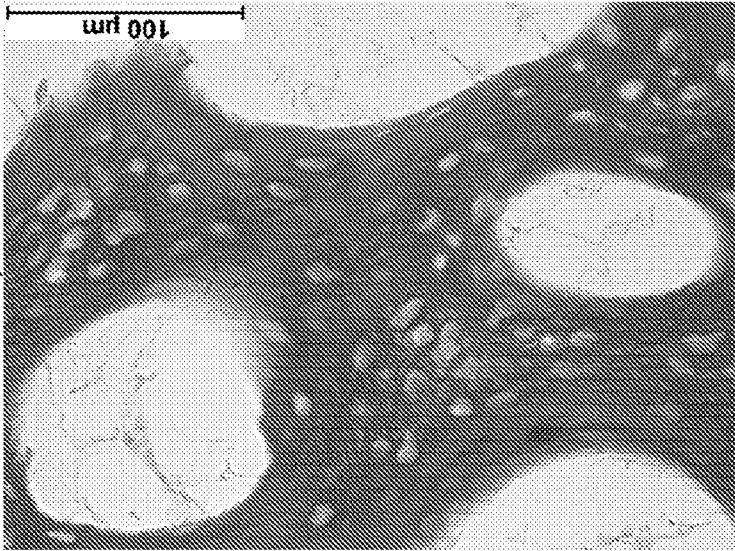


Figure 29B

2900

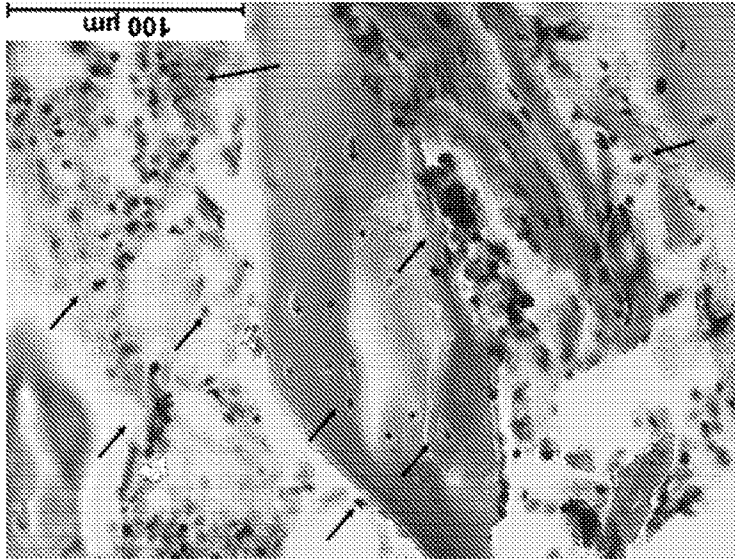
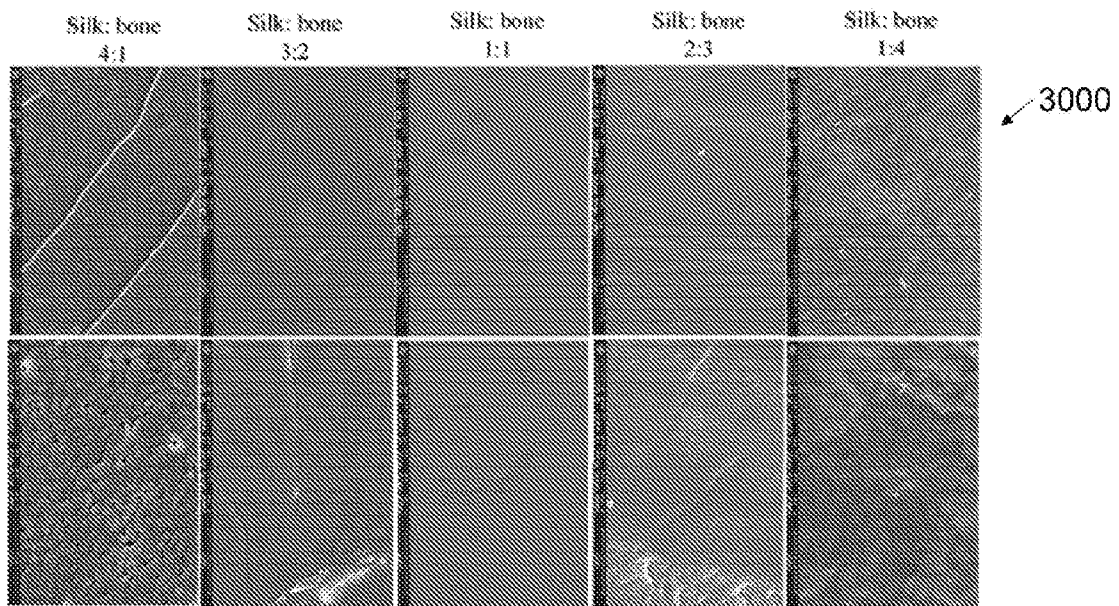
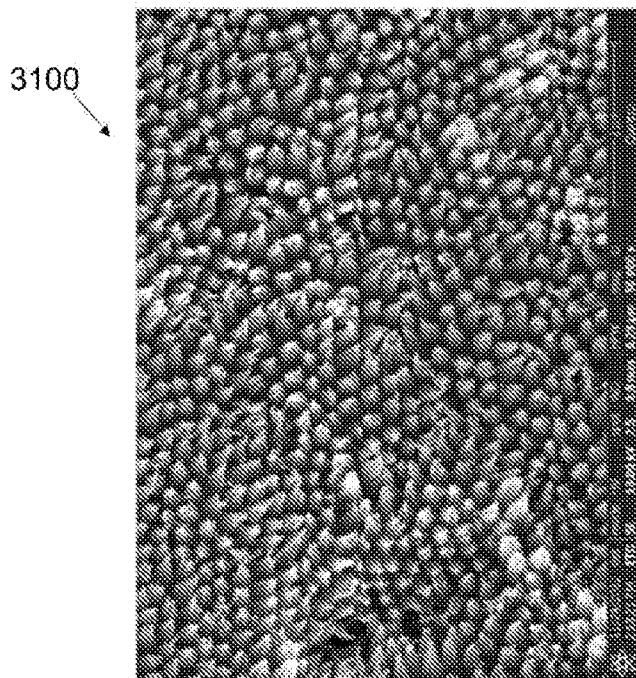
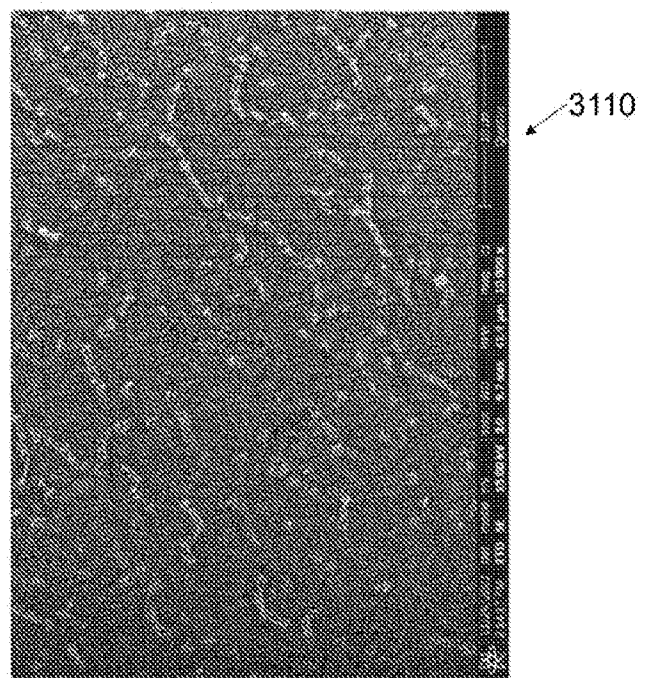


Figure 29A

**Figure 30****Figure 31A****Figure 31B**

3210 →

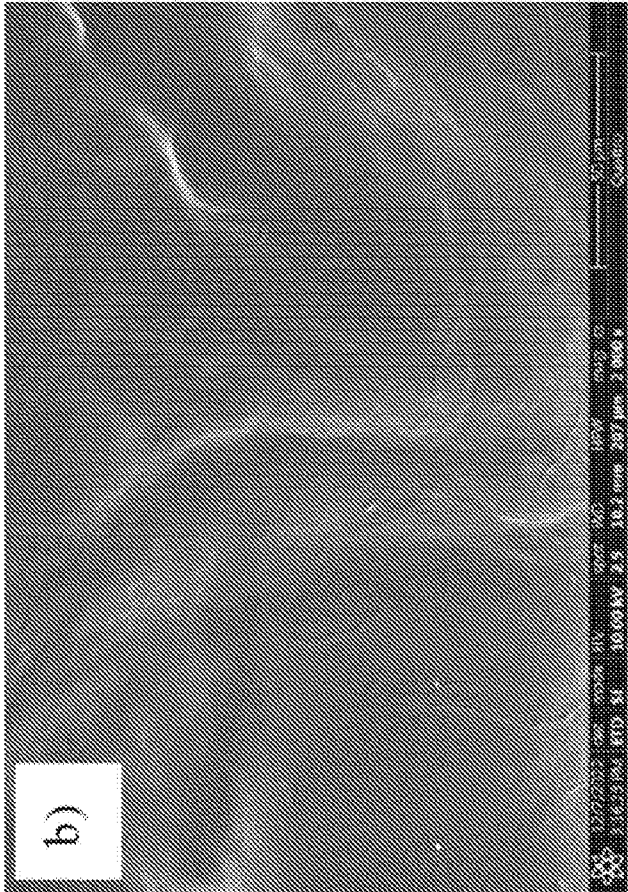


Figure 32B

3200 →

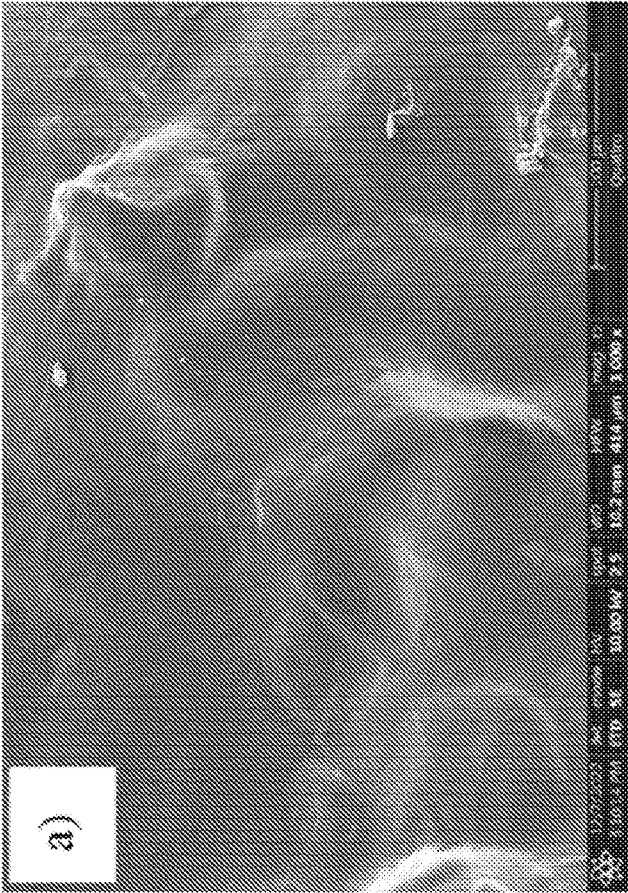


Figure 32A

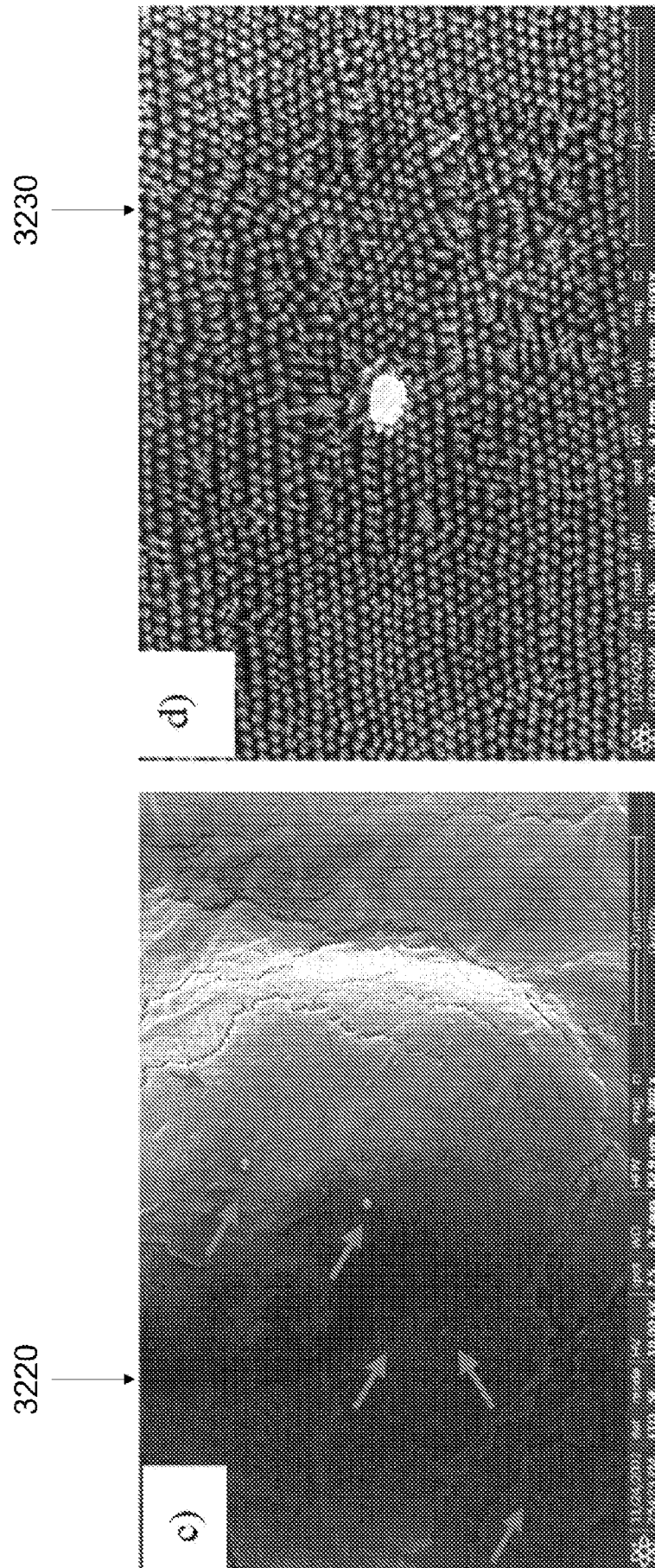


Figure 32C

Figure 32D

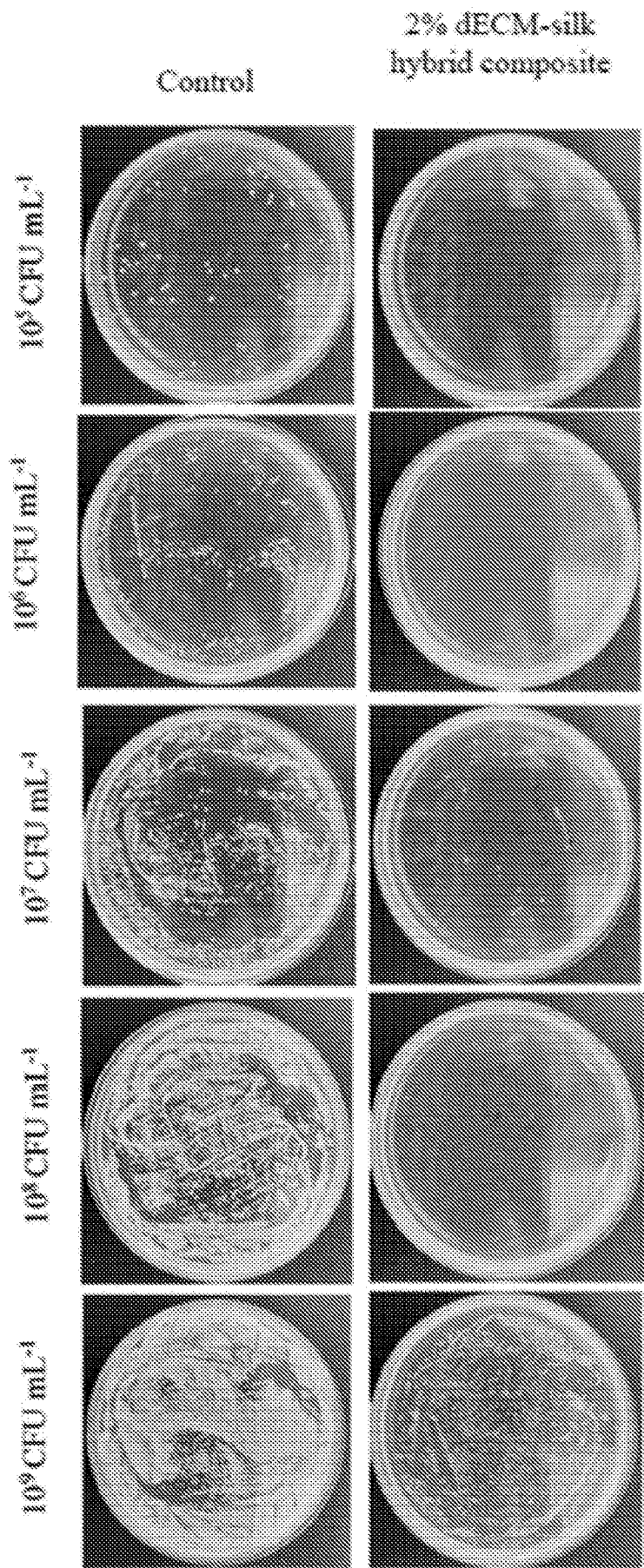
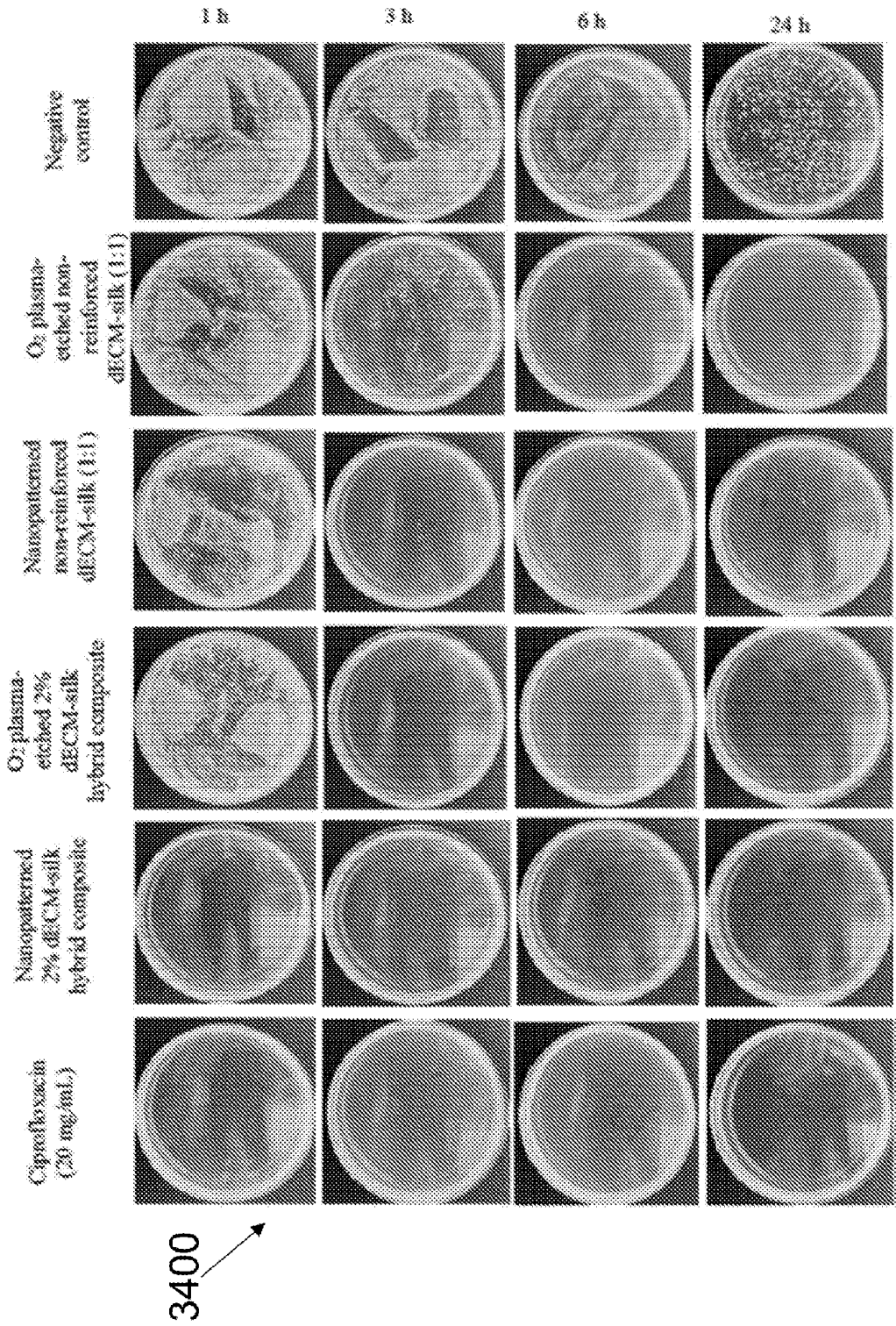


Figure 33

3300



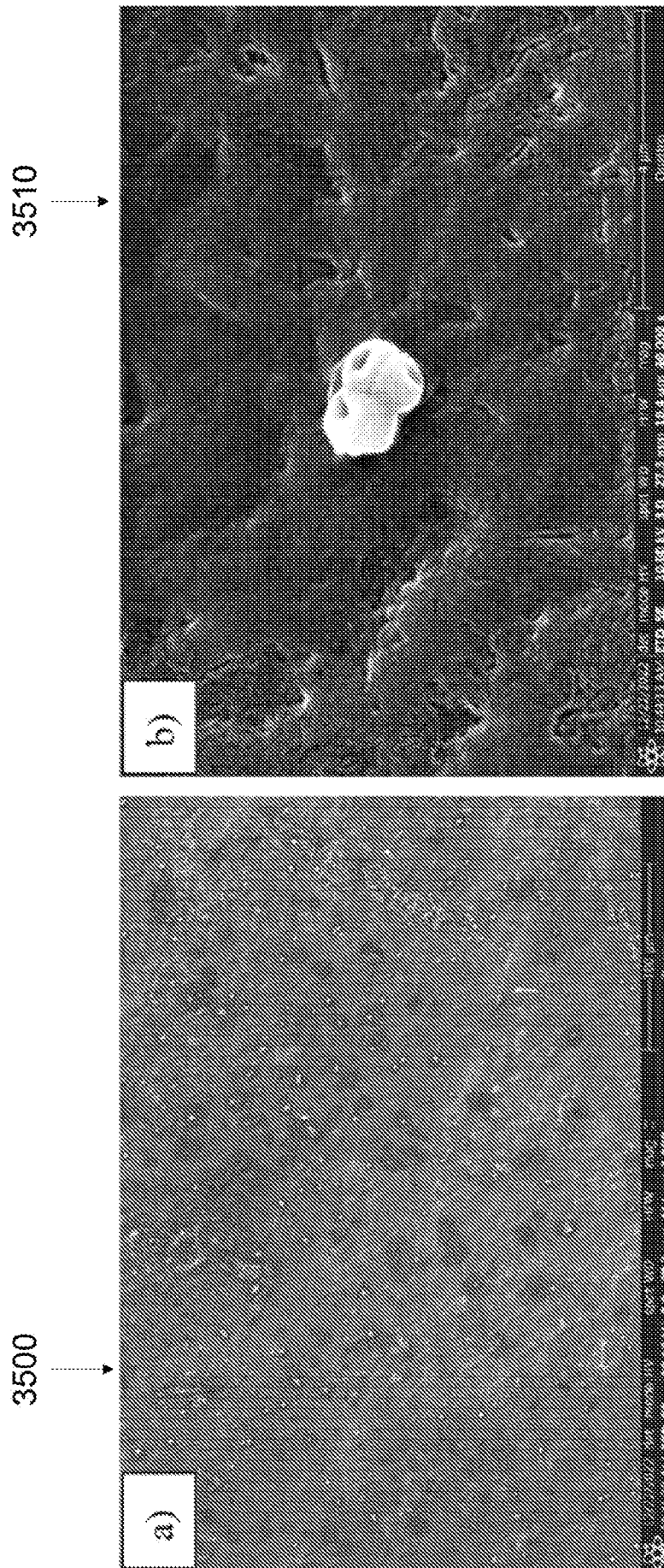


Figure 35B

Figure 35A

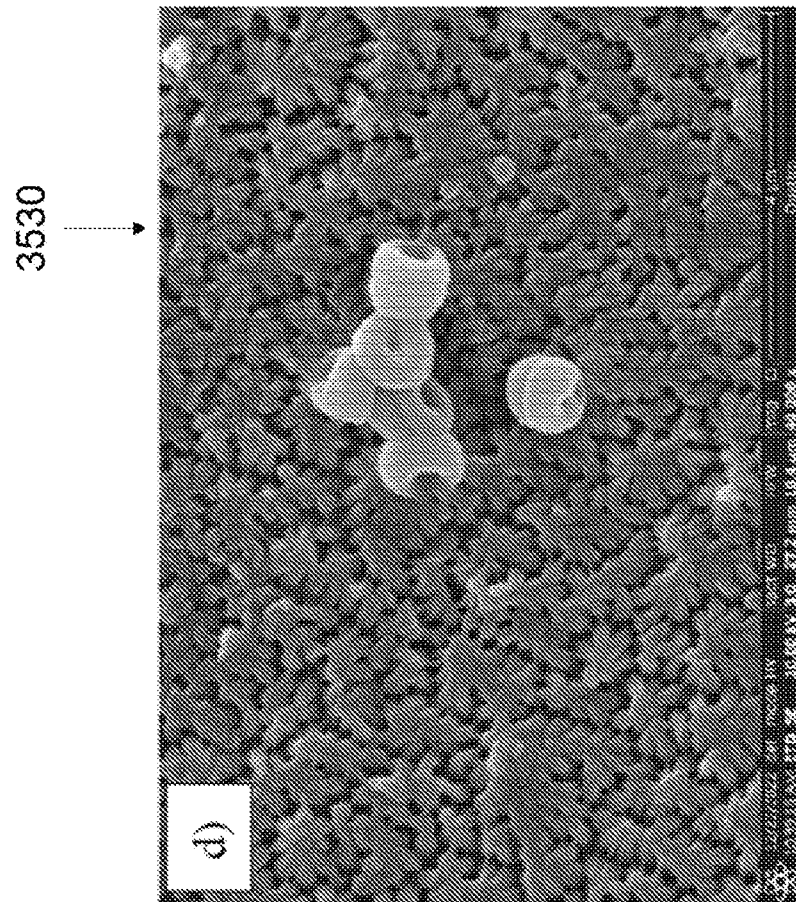


Figure 35D

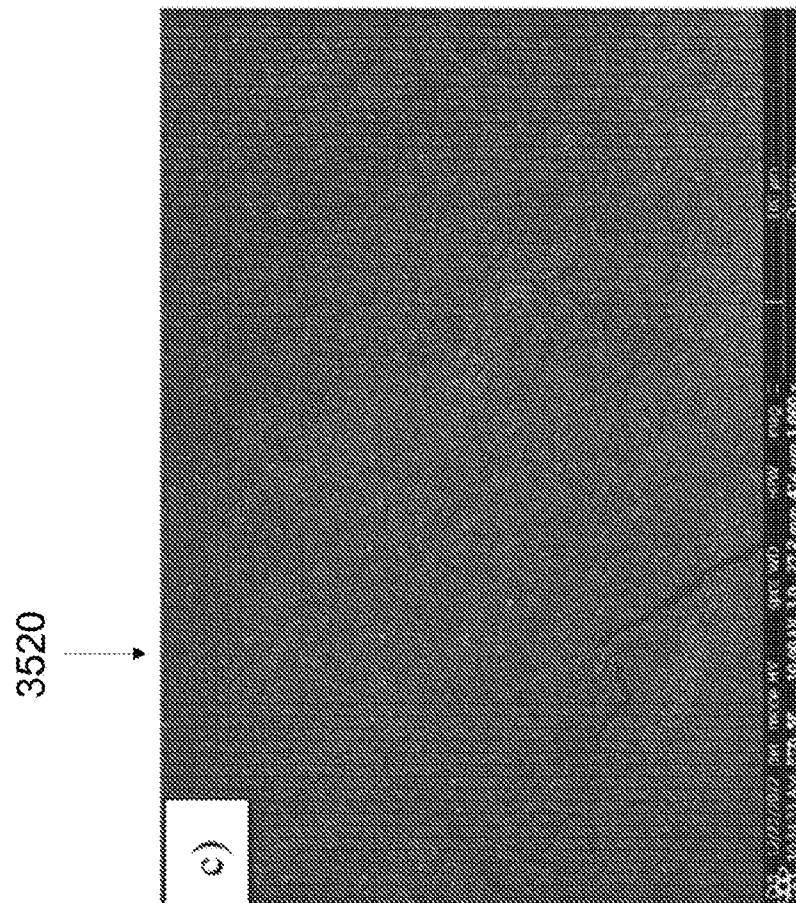


Figure 35C

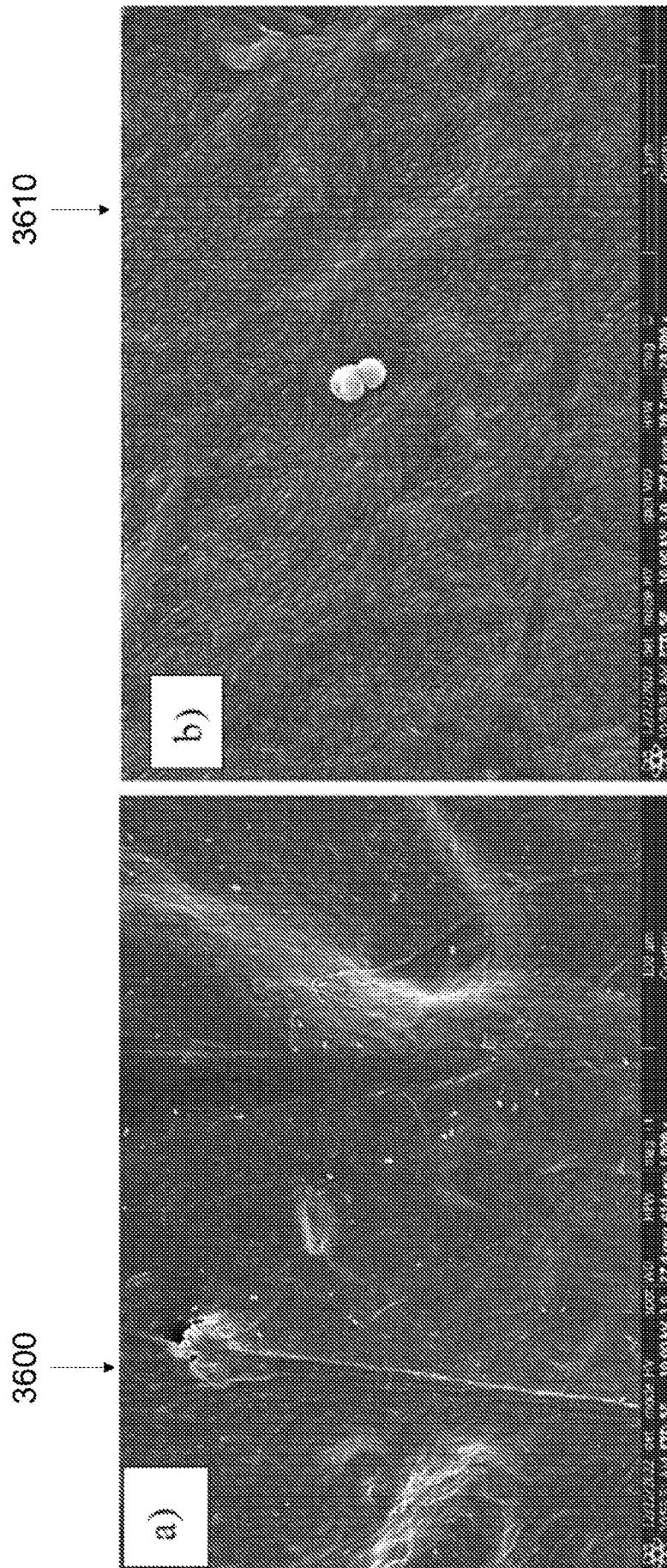


Figure 36B

Figure 36A

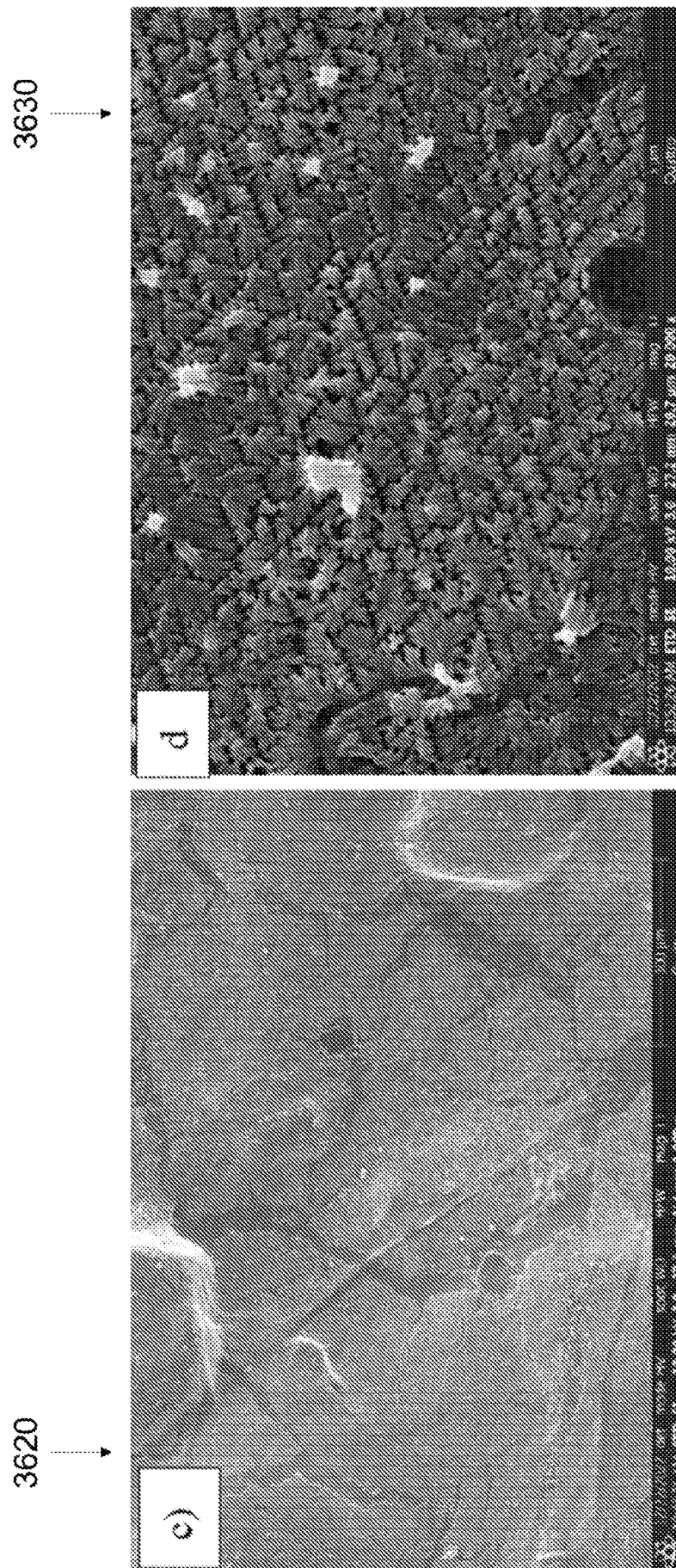


Figure 36D

Figure 36C

3710

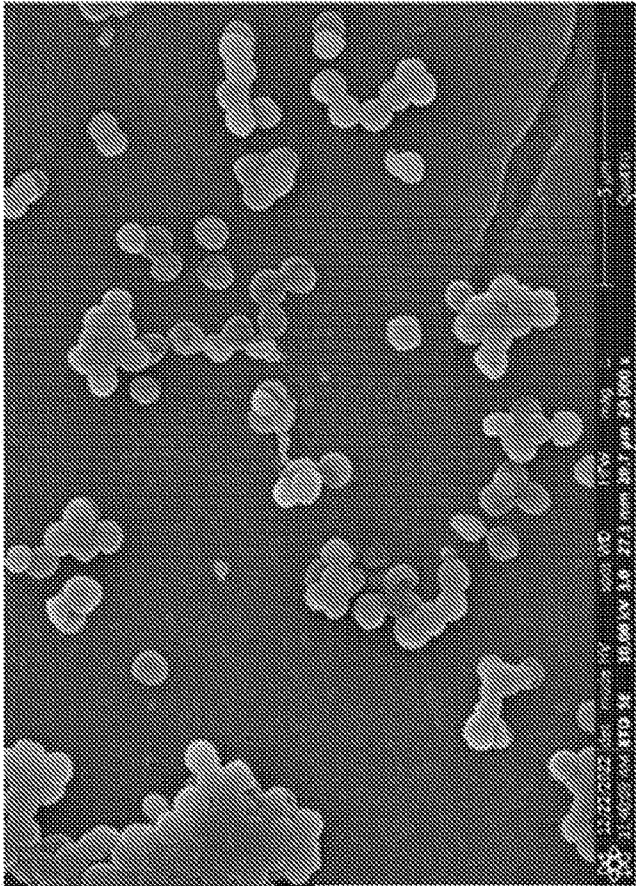


Figure 37B

3700

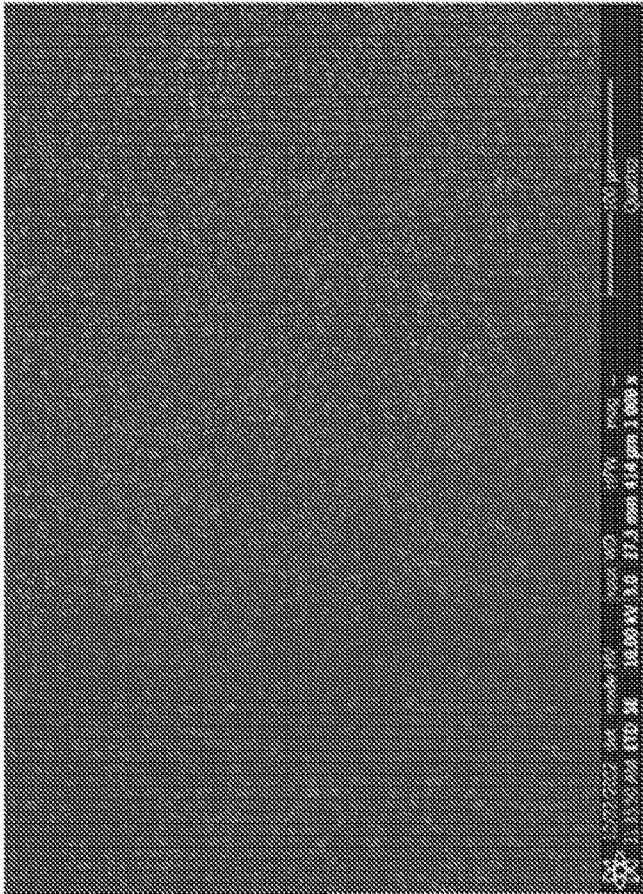


Figure 37A

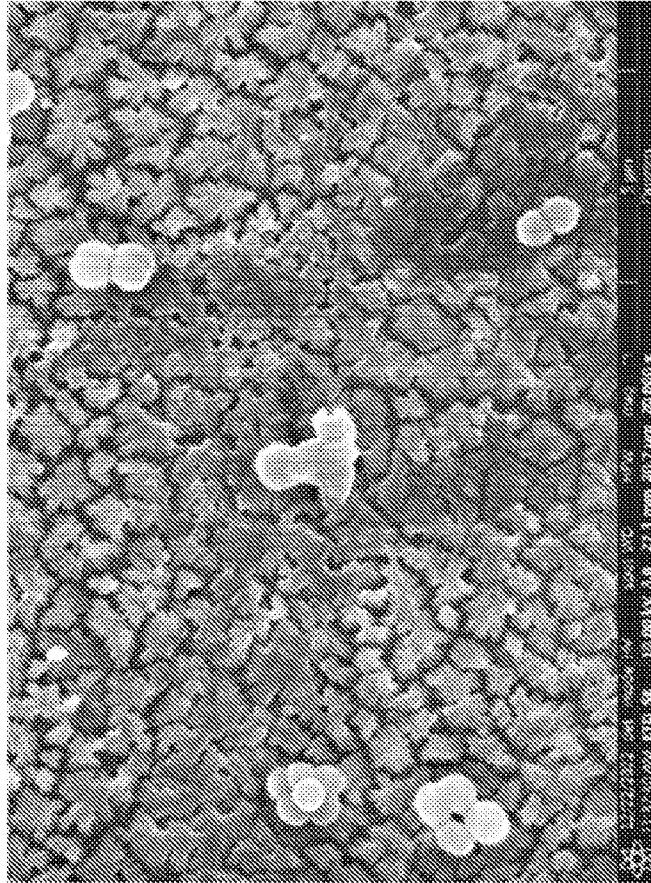
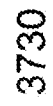


Figure 37D

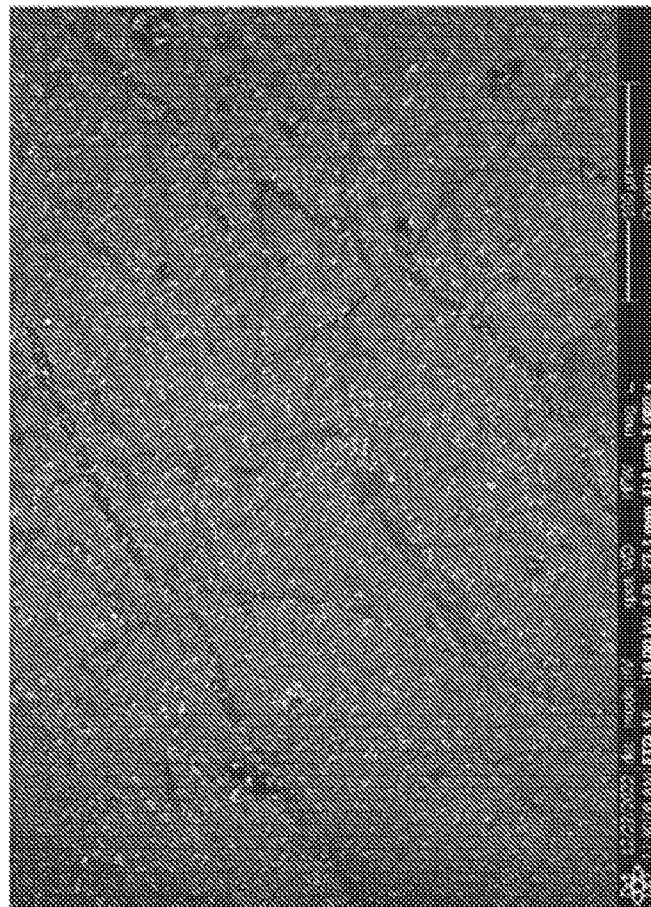


Figure 37C

3800

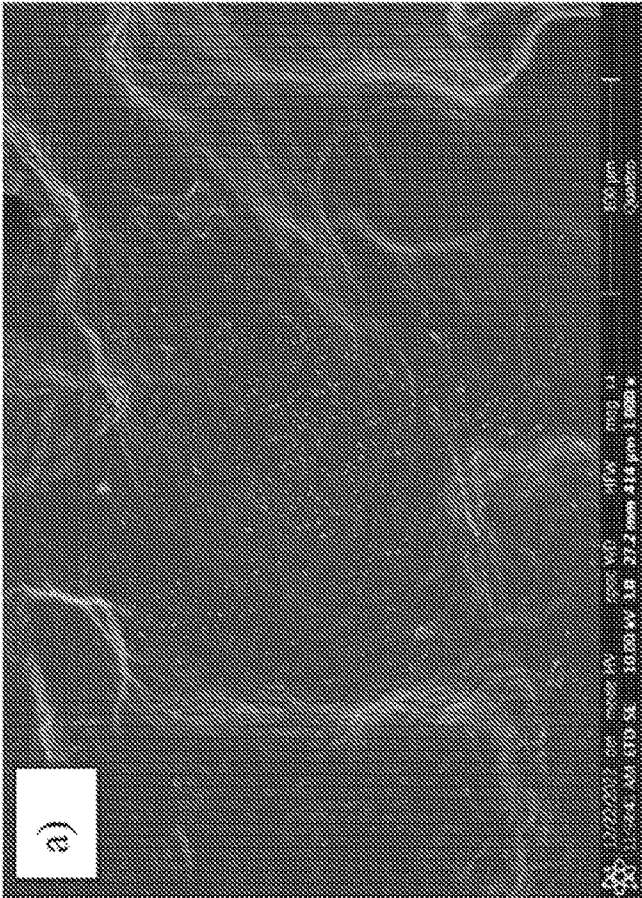


Figure 38A

3810

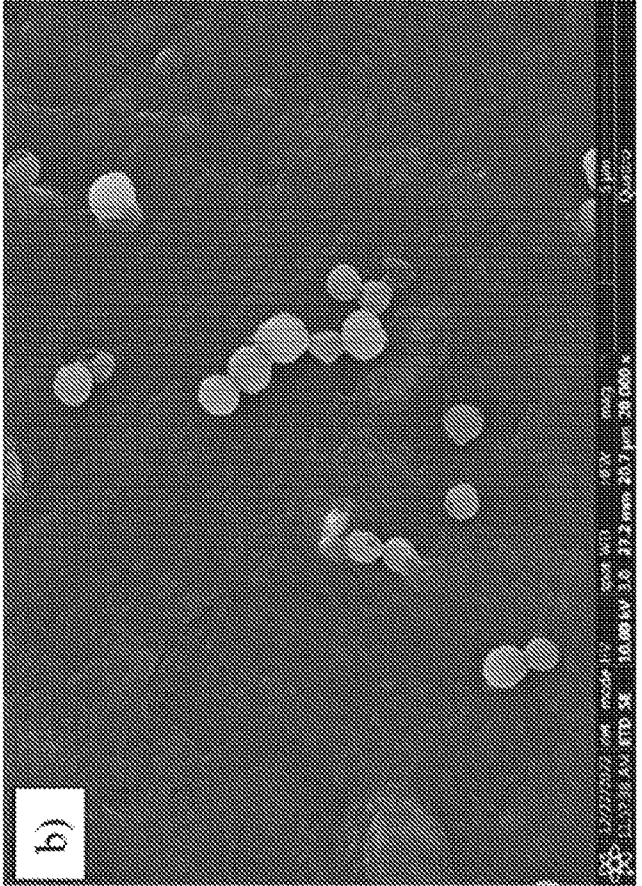


Figure 38B

3830

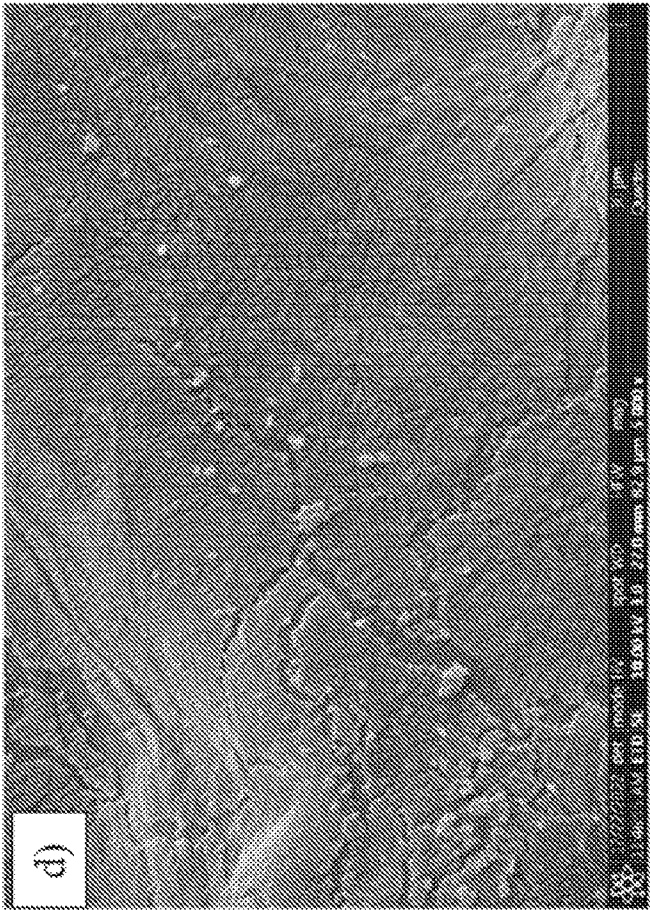


Figure 38D

3820

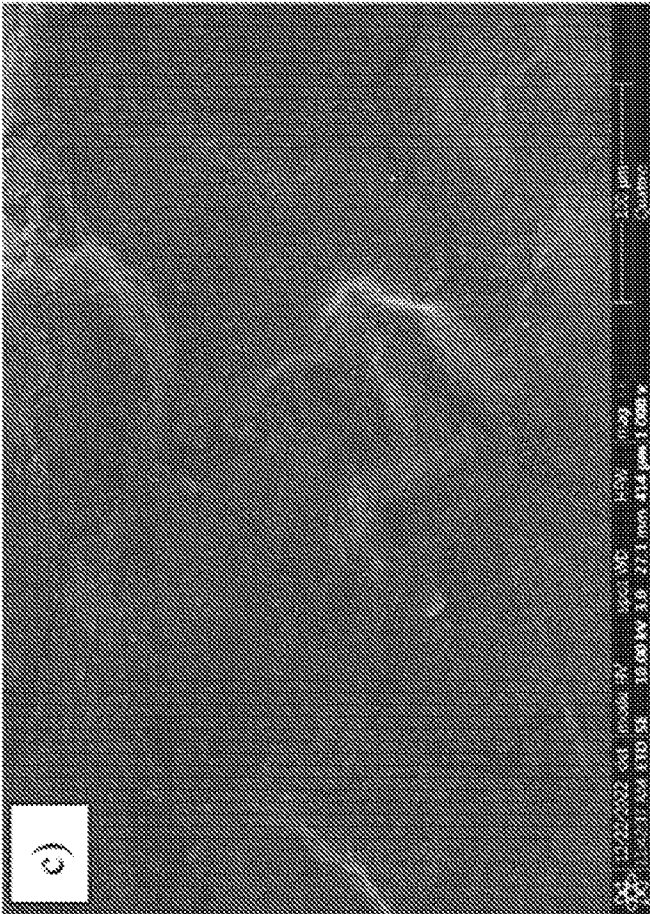


Figure 38C

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2024/088519

A. CLASSIFICATION OF SUBJECT MATTER

A61L27/14(2006.01)i; A61K38/19(2006.01)i; A61K38/18(2006.01)i; A61K35/32(2015.01)i

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)

IPC: 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)

CNTXT, WPABSC, WPABS, DWPI, ENTXT, CNKI: tissue regeneration, decellularized extracellular matrix, antibacterial, polymer, silk fibroin, chitosan, succinimide ester

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2005013872 A1 (BOSTON SCIENT SCIMED INC) 20 January 2005 (2005-01-20) claims 1-65, description paragraphs 74-81	1-13, 15-16, 18-2 5, 27, 29-30, 32-42
X	CN 105194734 A (YANTAI HAIAN DRUG DEV CO LTD) 30 December 2015 (2015-12-30) claims 1-5	1-13, 15-16, 18-2 5, 27, 29-30, 32-42
X	JP 2019076093 A (ISE PROFESSIONAL TESTING & CONSULTING SERVICES INC) 23 May 2019 (2019-05-23) claims 1-33, description paragraph 0090	1-13, 15-16, 18-2 5, 27, 29-30, 32-42
X	US 2005013870 A1 (Toby Freyman et al.) 20 January 2005 (2005-01-20) claims 1-41, description paragraph 150	1-13, 15-16, 18-2 5, 27, 29-30, 32-42
X	CN 105246495 A (ISE PROFESSIONAL TESTING & CONSULTING) 13 January 2016 (2016-01-13) claims 1-32, description paragraph 94	1-13, 15-16, 18-2 5, 27, 29-30, 32-42
X	US 2014099330 A1 (CMAT) CORMATRIX CARDIOVASCULAR INC et al. 10 April 2014 (2014-04-10) claims 1-16, description paragraphs 103-104	1-13, 15-16, 18-2 5, 27, 29-30, 32-42



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

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

“D” document cited by the applicant in the international application

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

“L” document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

“O” document referring to an oral disclosure, use, exhibition or other means

“P” document published prior to the international filing date but later than the priority date claimed

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

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

“Y” document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

“&” document member of the same patent family

Date of the actual completion of the international search

26 June 2024

Date of mailing of the international search report

11 July 2024

Name and mailing address of the ISA/CN

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Telephone No. (+86) 62084695

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2024/088519

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CN 101161293 A (Hu Shengshou et al.) 16 April 2008 (2008-04-16) the whole document	1-42

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2024/088519

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

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

1. ☒ Claims Nos.: **42**
because they relate to subject matter not required to be searched by this Authority, namely:

Claim 42 claims a method of repairing skin or bone tissue in a subject, the method comprising applying the dECM composition of claim 20 to the skin or bone tissue of the subject where tissue repair is required. The subject matter of claim 42 relates to a method of treatment of the human or animal body by therapy and therefore does not warrant an international search according to the criteria set out in Rule 39.1(iv). The subject matter of reasonable expectation is that “a manufacturing method of products for repairing skin or bone tissue in a subject, the method comprising applying the dECM composition of claim 20 to the skin or bone tissue of the subject where tissue repair is required.”
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/CN2024/088519

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
US	2005013872	A1	20 January 2005	DE	602004019337	D1	19 March 2009
				US	7326571	B2	05 February 2008
				WO	2005009498	A2	03 February 2005
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				EP	1654015	B1	04 February 2009

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				US	2015352257	A1	10 December 2015
				US	10617790	B2	14 April 2020
				US	2020188557	A1	18 June 2020
				US	11660376	B2	30 May 2023
				IL	239828	A0	31 August 2015
				IL	239828	B	31 May 2021
				US	2023302198	A1	28 September 2023
				KR	20150138157	A	09 December 2015
				KR	102284982	B1	03 August 2021
				CA	2897662	A1	17 July 2014
				CA	2897662	C	26 July 2022
				EP	2943209	A1	18 November 2015
				EP	2943209	A4	26 October 2016
				EP	2943209	B1	24 February 2021
				EP	2943209	B8	07 April 2021
				US	2019255217	A1	22 August 2019

US	2005013870	A1	20 January 2005	EP	1648533	A1	26 April 2006
				WO	2005009497	A1	03 February 2005
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				US	2009138074	A1	28 May 2009

CN	105246495	A	13 January 2016	WO	2014110269	A1	17 July 2014
				HK	1219907	A1	21 April 2017
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				JP	6606429	B2	13 November 2019
				US	2015352257	A1	10 December 2015
				US	10617790	B2	14 April 2020
				US	2020188557	A1	18 June 2020
				US	11660376	B2	30 May 2023
				IL	239828	A0	31 August 2015
				IL	239828	B	31 May 2021
				US	2023302198	A1	28 September 2023
				KR	20150138157	A	09 December 2015
				KR	102284982	B1	03 August 2021
				CA	2897662	A1	17 July 2014
				CA	2897662	C	26 July 2022
				JP	2019076093	A	23 May 2019

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/CN2024/088519

Patent document cited in search report				Publication date (day/month/year)		Patent family member(s)	Publication date (day/month/year)
				EP	2943209	A1	18 November 2015
				EP	2943209	A4	26 October 2016
				EP	2943209	B1	24 February 2021
				EP	2943209	B8	07 April 2021
				US	2019255217	A1	22 August 2019
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				CA	2887350	A1	17 April 2014
				IL	238066	A0	31 May 2015
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				SG	11201502714	TA	28 May 2015
				WO	2014058587	A1	17 April 2014
				HK	1207557	A1	05 February 2016
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				EP	2903560	A4	25 May 2016
CN	101161293	A	16 April 2008	None			