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(54) Title: MAMMALIAN CELL-COMPATIBLE FREE-STANDING NANOMEMBRANES

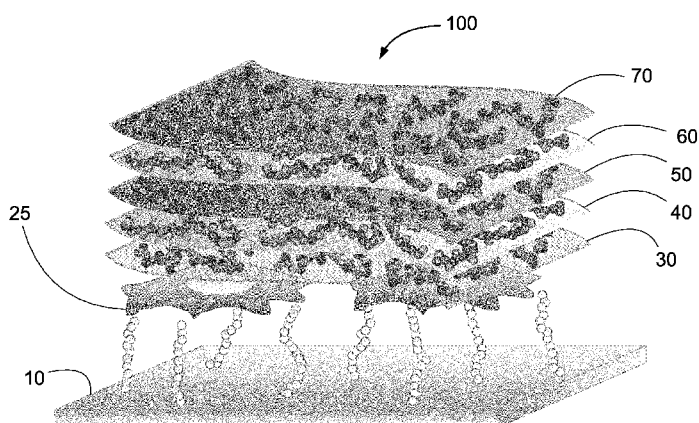


Fig. 7

(57) Abstract: A method for preparing a layered mammalian-cell compatible nanomembrane, comprising the steps of: (i) grafting a temperature-responsive substrate onto a support substrate having cleaned surfaces; (ii) culturing a layer of selected mammalian cells onto the surface of the temperature-responsive substrate; (iii) depositing a layer of gelatin onto the mammalian cells; (iv) depositing a layer of a cationic polyelectrolyte solution onto the layer of gelatin; (v) depositing a layer of an anionic polyelectrolyte solution onto the cationic polyelectrolyte layer; and (vi) storing the layered mammalian-cell compatible nanomembrane in a fluid storage medium at a temperature wherein the cell layer remains attached to the temperature-responsive substrate. The layered mammalian-cell compatible nanomembrane can be detached from the temperature-responsive substrate by adjusting the temperature of the fluid storage medium to a second temperature. One or more mammalian cell regulatory compounds may be added to the cationic polyelectrolyte solution and/or the anionic polyelectrolyte solution.



TITLE: MAMMALIAN CELL-COMPATIBLE FREE-STANDING  
NANOMEMBRANES

TECHNICAL FIELD

The present disclosure pertains to free-standing synthetic nanomembranes. More  
5 particularly, the present disclosure pertains to free-standing synthetic nanomembranes that  
are compatible with mammalian tissue systems.

BACKGROUND

Mammalian tissues and organs comprise combinations of cells and extracellular  
matrix. Organs generally comprise a continuous layer of epithelial cells, also referred to as  
10 the epithelium, under which is disposed a basement membrane comprising a thin sheet of  
fibers. The three-dimensional area encompassed by the epithelium and basement membrane  
is referred to as the interstitial space and generally comprises cells, fluids, polysaccharides  
gels, and various types of proteins. The term extracellular matrix (ECM) is commonly used to  
describe the combination of the basement membrane and the interstitial matrix. Due to its  
15 diverse nature and composition, the ECM can serve many functions, such as providing  
structural support to organs and tissues, segregating adjacent tissues, and regulating  
intercellular communication. In addition, the ECM sequesters a wide range of cellular growth  
factors. Physiological stresses resulting from environmental or physiological cues trigger  
protease activities that cause localized releases of cellular growth factors resulting in rapid  
20 and local growth factor-mediated activation of cellular functions. The ECM has essential  
roles in normal growth processes, wound healing, and abnormal processes such as fibrosis.

Numerous methods have been disclosed for preparation of cell sheets and  
nanomembrane complexes from selected isolated mammalian cells, for assessment of their  
potential for use in tissue reconstruction. For example, Ohashi et al. (2007, *Engineering*  
25 *functional two- and three-dimensional liver systems in vivo using hepatic tissue sheets*.  
*Nature Medicine* 13(7):880-885) and Yamato et al. (2007, *Temperature-responsive cell*  
*culture surfaces for regenerative medicine with cell sheet engineering*. *Prog. Poly. Sci.* 32(8-  
9):1123-1133) disclosed methods for preparing layered cell sheets. Ito et al. (2004, *Tissue*  
*engineering using magnetite nanoparticles and magnetic force: Heterotypic layers of*  
30 *cocultured hepatocytes and endothelial cells*. *Tissue Eng.* 10(5-6):833-840) and Ito et al.

(2005, *Novel methodology for fabrication of tissue-engineered tubular constructs using magnetite nanoparticles and magnetic force*. Tissue Eng. 11(9-10):1553-1561) provided processes for *in vitro* synthesis of magnetic liposomes. Ma et al. (2001, *A preliminary in vitro study on the fabrication and tissue engineering applications of a novel chitosan bilayer material as a scaffold of human neofetaf dermal fibroblasts*. Biomaterials 22(4):331-336) and  
5 Murugan et al. (2006, , *Nano-featured scaffolds for tissue engineering: A review of spinning methodologies*. Tissue Eng. 12(3):435-447) disclosed methods for preparation layered polymeric membranes. All of these methods typically culture functional tissues from the bottom up in 2-D dishes in cell culture media supplemented with hormones and cell  
10 regulators, or alternatively, in 3-D scaffolds that have been impregnated with hormones and cell regulators. The nanomembrane complexes or cell sheets fabricated by these methods can be made to mimic some mammalian tissues and have been found useful for systematic investigations of the effects of environmental and chemical cues on cellular functions.

However, while such fabricated nanomembrane complexes and cell sheets are useful  
15 for *in vitro* assays and assessments, they do not retain their mechanical stability over extended periods of time. Furthermore, it is not yet understood how to successful regulate cellular functions in synthetic nanomembranes and cell sheets, and how synchronize them with in situ tissues and organs.

## SUMMARY

20 The exemplary embodiments of the present disclosure pertain to systems and methods for preparing free-standing nanomembrane sheets and films that are compatible with mammalian cells and tissues and systems. The free-standing nanomembrane sheets comprise multiple layers of individually and sequentially deposited nanomembranes, comprising one or more materials. Each individual nanomembrane layer may be provided with one or more  
25 specific cell regulators that that are released in response to a cue for the purpose of eliciting a response in an underlying layer.

An exemplary method for preparing a free-standing nanomembrane sheet of the present disclosure generally comprises the steps of preparing a temperature-responsive substrate surface, culturing a cell suspension on the surface of the temperature-responsive  
30 substrate to produce a sheet of cells, sequentially depositing a plurality of nanomembranes layers onto the surface of the cell sheet to form a composite comprising the cell sheet and

layered nanomembranes, and detaching the composite from the temperature-responsive substrate.

## DESCRIPTION OF THE DRAWINGS

The present disclosure will be described in conjunction with reference to the following drawings in which:

Figs. 1(A)-1(C) illustrate an exemplary two step process for grafting a temperature-responsive poly(N-isopropylacrylamide) onto glass slides;

Fig. 2(A) is a 3D atomic force microscopy image of a glass slide with a self-assembled silane monomer formed by hydrolysis of 3-acryloxypropyltrimethoxysilane, and Fig. 2(B) is a 3D atomic force microscopy image of a glass slide after polymerization of poly(N-isopropylacrylamide) onto the silane monomer;

Fig. 3 is a chart showing the attenuated total reflectance spectrum of the glass slide from Fig. 2(B) generated by infrared light;

Fig. 4 is a perspective view of the distribution of temperature-responsive poly(N-isopropylacrylamide) substrate grafted onto the surface of a glass slide;

Fig. 5 is a perspective view of mammalian cells grown on the temperature-responsive poly(N-isopropylacrylamide) substrate shown in Fig. 4,

Fig. 6 is a perspective view of five layers of nanomembranes sequentially deposited onto the surface of the mammalian cell culture shown in Fig. 5;

Fig. 8 is a perspective view of a free-standing cell-nanomembrane sheet released from the glass substrate as conceptualized in Fig. 6;

Fig. 9 is a chart showing the contact angles of the glass slides after each of the treatments illustrated in Figs. 1-5;

Fig. 10 is a 3D atomic force microscopy image of a nanomembrane formed on a glass slide comprising a base layer of a self-assembled monolayer of silane monomer to which was polymerized poly(N-isopropylacrylamide) followed by sequential deposition of a gelatin layer, a chitosan layer, an alginate layer, and two more alternating layers of chitosan and

alginate;

Fig. 11 is a scanning electron microscopy image of the nanomembrane shown in Fig. 10;

Fig. 12(A) is a confocal laser scanning electron microscopy image of a live/dead assay on a glass slide comprising mammalian cells coated with an exemplary gelatin (chitosan/alginate)<sub>3</sub> nanofilm, while 12(B) a confocal laser scanning electron microscopy image of a live/dead assay on an exemplary gelatin (chitosan/alginate)<sub>3</sub> nanofilm after its detachment from a glass slide on which it was formed; and

Fig. 13(A) is a confocal laser scanning electron microscopy image of the surface of an exemplary nanomembrane stained with TO-PRO<sup>®</sup>-3 carbocyanine monomer nucleic acid stain, while 13(B) is a confocal laser scanning electron microscopy image of a cross-section of the nanomembrane stained with TO-PRO<sup>®</sup>-3 (TO-PRO is a registered trademark of Molecular Probes Inc., Eugene, OR, USA);

Fig. 14(A) is a confocal laser scanning electron microscopy image of the surface of an exemplary nanomembrane stained with NBD-phalloidin, while 14(B) is a confocal laser scanning electron microscopy image of a cross-section of the nanomembrane stained with NBD-phalloidin;

Fig. 15(A) is a confocal laser scanning electron microscopy image of the surface of an exemplary nanomembrane stained with rhodamine-labelled chitosan, while 15(B) is a confocal laser scanning electron microscopy image of a cross-section of the nanomembrane stained with rhodamine-labelled chitosan;

Fig. 16(A) is a confocal laser scanning electron microscopy image of the surface of an exemplary nanomembrane stained with TO-PRO<sup>®</sup>-3 stain, NBD-phalloidin , and rhodamine-labelled chitosan, while 16(B) is a confocal laser scanning electron microscopy image of a cross-section of the nanomembrane stained with TO-PRO<sup>®</sup>-3 stain, NBD-phalloidin , and rhodamine-labelled chitosan; and

Fig. 17 shows the effects of different culture systems on expression by mouse bone marrow stromal cells, of: (A) runt-related transcription factor 2(Runx2), (B) collagen type I(COL-I), (C) bone sialoprotein (BSP), and (D) osteopontin(OPN).

## DETAILED DESCRIPTION

The exemplary embodiments of the present disclosure pertain to systems and methods for preparing free-standing nanomembranes and films comprising extracellular matrix sheets, that are compatible with mammalian cells and tissues and systems.

5           The exemplary methods generally comprise the steps of preparing a temperature-responsive substrate surface, culturing a sheet of selected mammalian cells on the surface of the substrate, then sequentially adding layers of selected nanomaterials, i.e., layer-by-layer. One or more cell regulators and/or other bioactive molecules can be incorporated into each nanolayer. However, it isn't necessary, if so desired, to incorporate cell regulators into each  
10 layer. After a nanomembrane sheet has been fabricated with the plurality of selected nanomembrane layers overlaying the cell sheet, the nanomembrane sheet is released from the temperature-responsive substrate surface into a cooled buffered solution. The transition of surface wettability at a lowered temperature allows the cell sheet to detach spontaneously from the grafted surfaces.

15           An exemplary method for fabricating a biomimetic nanomembrane comprises a first step of preparing a temperature-responsive substrate surface on a glass slide by first forming a self-assembled silane monolayer by hydrolyzing 3-acryloxypropyltrimethoxysilane (APTES) onto a clean glass surface. Then, the poly(N-isopropylacrylamide) (PNIPAM) is polymerized onto the silane monolayer to uniformly cover the glass surface with hydroxyl  
20 ions. The second step comprises immersing the PNIPAM-coated glass slide into dish containing a suitable nutrient solution and culturing therein a selected mammalian cell culture on the surface of the PNIPAM-coated glass slide. The third step comprises depositing a layer of Type A gelatin to cover the cell culture on the surface of PNIPAM-coated glass slide to preserve and maintain cell viability during the subsequent layer-by-layer deposition of  
25 additional layers of biocompatible polyelectrolytes exemplified by chitosan and alginate. Suitable concentrations of Type A gelatin include 0.01% w/v, 0.025% w/v, 0.05% w/v, 0.075% w/v, 0.10% w/v, 0.15% w/v, 0.20% w/v, 0.25% w/v, 0.30% w/v, 0.35% w/v, 0.40% w/v, 0.45% w/v, 0.50% w/v and therebetween. Suitable concentrations of chitosan or alginate or other such biocompatible polyelectrolytes include 0.01% w/v, 0.025% w/v, 0.05%  
30 w/v, 0.075% w/v, 0.10% w/v, 0.15% w/v, 0.20% w/v, 0.25% w/v, 0.30% w/v, 0.35% w/v, 0.40% w/v, 0.45% w/v, 0.50% w/v and therebetween. Steps one through three are preferably done at ambient temperature, for example about 18° C, about 20° C, about 22° C, about

24° C, about 26° C, about 28° C, about 30° C, and therebetween. The fourth step comprises placing the dish containing therein the glass slide with the fabricated nanomembrane cell sheet within the nutrient solution into a chilled environment for a period of time. The transition of surface wettability at lowered temperatures allows the fabricated nanomembrane cell sheet to detach spontaneously from the PNIPAM grafted onto the surface of the glass slide. Suitable temperatures for providing the chilled environment are exemplified by about 3° C, about 4° C, about 5° C, about 6° C, about 8° C, about 10° C, about 12° C, about 14° C, about 16° C, and therebetween. The detached fabricated nanomembrane cell sheet can be stored in fresh nutrient solution under refrigeration until required for use.

If so desired, one or more cellular regulatory compounds and/or antibiotic and/or nutrients and/or micronutrients may be added to each layer of biocompatible polyelectrolytes before it is deposited onto the cellular matrix. Suitable regulatory compounds are exemplified by bone morphogenetic proteins, dexamethasone, ascorbic acid,  $\beta$ -glycerol phosphate, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), tissue inhibitor of metalloproteinase (TIMP), neuropilin-1 (NRP-1), and the like. Suitable concentrations for incorporating such regulators into a polyelectrolyte solution are from a range of about 5 ng to about 200 ng/ml and therebetween. Suitable antibiotics are exemplified by aminoglycosides exemplified by tobramycin, gentamycin, neomycin, gentamicin, streptomycin, and the like; azoles exemplified by fluconazole, itraconazole, and the like;  $\beta$ -lactam antibiotics exemplified by penams, cepheems, carbapenems, monobactams,  $\beta$ -lactamase inhibitors, and the like; cephalosporins exemplified by cefacetrile, cefadroxy, cephalexin, cephalosin, cefprozil, cefbuparazone, and the like; chloramphenicol; clindamycin; fusidic acid; glycopeptides exemplified by vancomycin, teicoplanin, ramoplanin, and the like; macrolides exemplified by azithromycin, clarithromycin, dirithromycin, erythromycin, spiramycin, tylosin, and the like; metronidazole; mupirocin; penicillins exemplified by benzylpenicillin, procaine benzylpenicillin, benzathine benzylpenicillin, phenoxymethylpenicillin, and the like; polyenes exemplified by amphotericin B, nystatin, natamycin, and the like; quinolones exemplified by ciprofloxacin, ofloxacin, dan ofloxacin, and the like; rifamycins exemplified by rifampicin, rifabutin, rifapentine, rifaximin, and the like; sulfonamides exemplified by sulfacetamide, sulfadoxine, and the like; tetracyclines exemplified by doxycycline, minocycline, tigecycline, and the like; and trimethoprim, among others. Suitable concentrations for incorporating such antibiotics into a polyelectrolyte solution are from a range of about from about 0.001% to about 10.0%

(w/w or w/v or v/v) and therebetween. Suitable micronutrients are exemplified by Vitamin A, the group Vitamin B, Vitamin C, Vitamin D, Vitamin E, Vitamin K, carotinoids, and the like. Suitable concentrations for incorporating such antibiotics into a polyelectrolyte solution are from a range of about from about 0.001% to about 0.1% (w/w or w/v or v/v) and  
5 therebetween.

The exemplary free-standing nanomembrane cell sheets and films prepared with the exemplary methods disclosed herein form biomimetic layered tissues that have increased mechanical stability relative to nanomembranes prepared by previous disclosed methods available in the public domain. It is proposed that the free-standing nanomembrane cell sheets  
10 and films prepared as disclosed herein, are useful for regulating cell functions *in situ* in tissue engineering applications. The present free-standing nanomembrane cell sheets and films are also useful for systematic investigations of the effects of environmental and chemical cues on cellular functions.

The following examples are provided to enable a better understanding of the  
15 disclosure described herein.

### **EXAMPLES**

Example 1: Preparation of glass slides with a temperature-responsive substrate grafted onto their surfaces

Glass slides were sequentially rinsed with: (i) chloroform, (ii) ethanol and (iii)  
20 distilled water. Then, the slides were immersed for one hour in a solution referred to herein as a “piranha solution”, comprising a 70:30 mixture of concentrated sulfuric acid (98 wt. %) and hydrogen peroxide (30 wt. %) thereby producing a layer of hydroxyl ions on the surfaces of the glass slides (Fig. 1(A)). The glass slides were the washed with copious amounts of a distilled water:ethanol solution, and dried in a vacuum oven for 24 hours.

25 The hydroxylated glass wafers were placed into a 3 % (w/v) toluene solution of 3-acryloxypropyltrimethoxysilane (APTES) and refluxed under nitrogen atmosphere for 24 h to graft the APTES onto the hydroxyl ions as shown in Fig. 1(B). Then, the glass slides were ultra-sonicated in toluene for 5 min. Then they were rinsed with acetone, ethanol and dried in vacuum.

Then, poly(N-isopropylacrylamide) (PNIPAM) was grafted onto the slides by immersion of slides into a 2% solution (v/v) of the photo-initiator (1,1'-Azobis(cyclohexanecarbonitrile)). The PNIPAM was spin-coated onto the 3-acryloxypropyltrimethoxysilane-modified glass wafers at 800 rpm for 30 sec. The slides were irradiated through a photomask for 5 min by a 365 nm-UV light source (80mW/cm<sup>2</sup>). The PNIPAM-grafted glass wafers (Fig. 1(C)) were then rinsed thoroughly by sonication in solution of ethanol and distilled water, and dried in vacuum. Fig. 2 shows a perspective conceptual view of the distribution of PNIPAM grafted onto the surface of a glass slide.

The PNIPAM-modified glass surfaces were characterized after each of the above-noted steps by: (i) using a Kruss CA-A contact angle measuring system to determine contact angles on the slide surfaces, (ii) determining the attenuated total reflectance (ATR), and (iii) tapping-mode atomic force microscopy (AFM) following the procedures disclosed by Shin et al. (2011, *Photolabile micropatterned surfaces for cell capture and release*. Chem. Comm. 47(43):11942-11944). AFM images were acquired by using standard silicon TESP probes having a nominal spring constant of 50 N/m and a resonance frequency of 300 kHz.

The contact angle of water droplets on the glass surfaces prior to commencing the above-noted steps was 15°. After attaching a layer of hydroxyl ions onto the surfaces of the glass slides with the piranha solution, the contact angle across the surfaces was uniformly 12°. The contact angles measured after grafting the APTES increased to 72°, and after grafting of the PNIPM, the contact angles were reduced to 62°.

Fig. 2(A) is a 3-D AFM image of a glass slide with a self-assembled silane monomer formed by hydrolysis of APTES. Fig. 2(B) is a 3-D AFM image of a glass slide after polymerization of PNIPAM onto the silane monomer. Fig. 3 is a chart showing the attenuated total reflectance spectrum of the glass slide from Fig 2(B) generated by infrared light.

**Example 2:** Culturing a layer of mammalian cells onto the PNIPAM grafted onto the surface of a glass slide

Exemplary free-standing nanomembrane sheets were prepared with two types of mammalian cells.

First, human myoblast C2C12 cells were seeded onto PNIPAM-grafted slides prepared as disclosed in Example 1, and were cultured in Dulbecco's modified Eagle's

medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) plus 100 units/ml penicillin until the cell layer developed a density of about  $2 \times 10^6$  cells/ml. The seeded slides were incubated at 37°C in a humidified incubator containing 5% CO<sub>2</sub> until the cell layer developed a density of about  $2 \times 10^6$  cells/ml. The medium was changed every three days until  
5 the cell density target was achieved.

Second, mouse bone marrow mesenchymal stem cells (BMSCs) were purchased from the American Type Culture Collection (ATCC, USA). Mouse BMSCs were seeded onto PNIPAM-grafted slides prepared as disclosed in Example 1, and were cultured in DMEM with high glucose e.g., about 4,500 mg/L, until the cell layer developed a density of about  
10  $2 \times 10^6$  cells/ml. This medium was supplemented with 10% FBS, 100 unit/ml penicillin, and 100 ug/ml streptomycin. The seeded slides were incubated at 37°C in a humidified incubator containing 5% CO<sub>2</sub>. The medium was changed every three days until the cell density target was achieved.

Example 3: Layer-by-layer fabrication of an exemplary free-standing nanomembrane sheet

15 Fig. 4 shows a perspective conceptual view of the distribution of PNIPAM 20 grafted onto the surface of a glass slide 10 according to the method disclosed in Example 1. Fig. 5 shows a perspective conceptual view of a layer of mammalian cells 25 cultured onto the surface of the PNIPAM 20 grafted onto glass slide.

The first biocompatible biopolymer layer 30 deposited onto the layer of mammalian  
20 cells 25 is type A gelatin (Fig 6). A 0.5 % solution (w/v) of type A gelatin (Sigma-Aldrich) was prepared by dissolving the polymer in PBS at pH 7.4. Prior to depositing the layer of type A gelatin, the glass slide grafted with PNIPAM onto which was cultured a layer of mammalian cells, was rinsed with PBS warmed to 37° C, and then was placed into a glass dish. Then, the gelatin layer 30 was deposited on the cell layer 20 by carefully adding 1 ml of  
25 the 0.5% gelatin solution using a pipette. The glass dish was placed for 10 min into an incubator held at for 37° C. After the 10-min incubation, excess gelatin solution was removed by aspiration.

The next step was deposition of a cationic polyelectrolyte layer 40 on top of the gelatin layer 30. A 0.1% chitosan (medium molecular weight, Sigma-Aldrich) solution was  
30 prepared by dissolving the polymer in 1 % (v/v) acetic acid and maintained at pH between

6.1 and 6.3. The chitosan solution was warmed to 37° C, and then carefully deposited onto the gelatin layer 30 using a pipette, after which, the glass plate containing the glass slide 10 into the 37° C incubator. After a 5-min incubation period, excess chitosan solution was removed by aspiration.

5           The next step was deposition of an anionic polyelectrolyte layer 50 on top of the cationic layer 40. A 0.1 % (w/v) solution of alginic acid (Sigma-Aldrich) was prepared in PBS and maintained at the physiological pH (7.4). The alginic acid solution was warmed to 37° C, and then carefully deposited onto the chitosan layer 40 using a pipette, after which, the glass plate containing the glass slide 10 into the 37° C incubator. After a 5-min incubation  
10       period, excess alginic acid solution was removed by aspiration.

          An additional layer of cationic solution 60 and then a layer of anionic solution 70 was sequentially deposited onto the slide 10 thereby forming the exemplary nanomembrane sheet 100. After the last layer was deposited, the nanomembrane sheet was rinsed with PBS pre-warmed to 37° C, and then immersed in an appropriate culture medium and stored in a 37° C  
15       incubator until required for use. For example, a nanomembrane sheet that was fabricated over a layer of human C2C12 cells was stored in DMEM supplemented with 10 % FBS. A nanomembrane sheet that was fabricated over a layer of mouse BMSCs cells was stored in DMEM supplemented with glucose.

          When needed, the nanomembrane sheet 100 can be removed from the glass slide 10  
20       by immersion of the glass slide into fresh cold PBS solution (e.g., about 4° C) for about 1 h during which time, the PNIPAM will detach from the mono-silane layer on the slide 10 (Fig. 7) and the nanomembrane sheet 100 will float freely in the PBS (Fig. 8). Alternatively, it is possible to remove the nanomembrane sheet 100 from the glass slide 10 simply by removing the glass slide 10 from storage in the 37° C incubator followed by its immersing into fresh  
25       PBS solution at ambient room temperature, for example at a temperature from a range of about 18° C to about 28° C, for about 2h to about 3 h.

          It is to be noted that the above method teaches fabrication of a nanomembrane sheet comprising a base layer of mammalian cells 25 over which is deposited a layer of type A gelatin 30 followed by two sets of alternating layers of cationic polyelectrolytes 40, 60 and  
30       anionic polyelectrolytes 50, 70. It is suitable to refer to such a nanomembrane as a “cell-gelatin-(chitosan/alginate)<sub>2</sub>” membrane. However, it is possible to fabricate nanomembranes

following the methods disclosed herein with three sets or four sets or more of alternating chitosan/alginate layers. Accordingly, it is suitable to refer to such nanomembranes as “cell-gelatin-(chitosan/alginate)<sub>3</sub>” membranes or “cell-gelatin-(chitosan/alginate)<sub>4</sub>” membranes respectively, or for convenience, as “cell-gelatin-(chitosan/alginate)<sub>x</sub>” membranes with “x” designating the number of chitosan/alginate sets.

Fig. 9 is a chart showing the contact angle of each layer deposited in a cell-gelatin-(chitosan/alginate)<sub>3</sub> membrane determined as outlined in Example 1. The contact angle of the cell layer was about 22°, and the contact angle increased to 25° after the application of a gelatin coating. The deposition of a chitosan layer on the gelatin increased the contact angle to about 45°. The following deposited alginate layer reduced the contact angle to around 20°. The final contact angle of the assembly was about 20°. Fig 10 is an AFM image of a free-standing cell-gelatin-(chitosan/alginate)<sub>3</sub> membrane after it was separated from its glass slide, while Fig. 11 is a scanning electron microscope image of the free-standing cell-gelatin-(chitosan/alginate)<sub>3</sub> membrane.

Example 4: Assessment of the compatibility of mammalian cells with the cationic polyelectrolyte layers and anionic polyelectrolyte layers

The viabilities of cells in mammalian cell layers that were coated during LBL assembly of gelatin-(chitosan-alginate)<sub>3</sub> membranes were assessed with a LIVE/DEAD® Viability/Cytotoxicity Kit sourced from Molecular Probes Inc. (Product # L3224; LIVE/DEAD is a registered trademark of Molecular Probes Inc., Eugene, OR, USA). Live and dead cells were identified on the basis of their membrane integrity and esterase activity. Nanomembranes fabricated over layers of human C2C12 cells were stored on glass slides and free-standing nanomembranes prepared as outlined in Example 3, were removed their storage media, rinsed with PBS, and then stained by addition of 2 µM calcein AM and 4 µM EthD-1 in PBS. Samples were incubated at 37° C for 10 min and observed using confocal laser scanning microscopy (CLSM). Fig 12(A) is a CLSM image of a nanomembrane attached to the glass slide on which it was fabricated. Fig, 12(B) is a CLSM image of a free-standing nanomembrane.

Free-standing nanomembranes fabricated over layers of BMSCs cells were prepared as outlined in Example 3. Samples of the free-standing nanomembranes were washed three times with cold PBS (about 4° C), after which, the cells comprising the cell layer were fixed

in 4% paraformaldehyde in PBS at ambient room temperature for 15 min. After fixation, the cells were permeabilized by 0.1% Triton X-100 in PBS for 10 min after which, the cells were washed three times with PBS. The cells were incubated in 10  $\mu$ M TO-PRO<sup>®</sup>-3 carbocyanine monomer nucleic acid stain for 20 min, followed by three washes with PBS (TO-PRO is a registered trademark of Molecular Probes Inc., Eugene, OR, USA). The films were then transferred to glass slides, covered with coverslips, and examined by confocal laser scanning microscopy. Figs. 13(A) and 13(B) are CLSM images of the surface and a cross-section, respectively, of a free-standing nanomembrane stained with TO-PRO<sup>®</sup>-3. The blue color is attributed to nuclei stained by TO-PRO<sup>®</sup>-3.

Free-standing nanomembranes fabricated over layers of BMSCs cells were prepared as outlined in Example 3. Samples of the free-standing nanomembranes were washed three times with cold PBS (about 4° C), after which, the cells comprising the cell layer were fixed in 4% paraformaldehyde in PBS at ambient room temperature for 15 min. After fixation, the cells were permeabilized by 0.1% Triton X-100 in PBS for 10 min after which, the cells were washed three times with PBS. The cells were incubated in 10  $\mu$ M phalloidin/1% (w/v) BSA solution for 20 min followed by three washes with PBS. The films were then transferred to glass slides, covered with coverslips, and examined by confocal laser scanning microscopy. Figs. 14(A) and 14(B) are CLSM images of the surface and a cross-section, respectively, of a free-standing nanomembrane stained with phalloidin. The green color is attributed to the F-actin in the cellular cytoskeleton stained by phalloidin.

Free-standing nanomembranes fabricated over layers of BMSCs cells were prepared as outlined in Example 3. Samples of the free-standing nanomembranes were washed three times with cold PBS (about 4° C), after which, the cells comprising the cell layer were fixed in 4% paraformaldehyde in PBS at ambient room temperature for 15 min. After fixation, the cells were permeabilized by 0.1% Triton X-100 in PBS for 10 min after which, the cells were washed three times with PBS. The cells were incubated in 1- $\mu$ M rhodamine-conjugated chitosan (Rh-chitosan) solution for 30 min followed by three washes with PBS. The films were then transferred to glass slides, covered with coverslips, and examined by confocal laser scanning microscopy. Figs. 16(A) and 16(B) are CLSM images of the surface and a cross-section, respectively, of a free-standing nanomembrane stained with rhodamine red. The red color is attributed to rhodamine-labelled proteins.

Free-standing nanomembranes fabricated over layers of BMSCs cells were prepared

as outlined in Example 3. Samples of the free-standing nanomembranes were washed three times with cold PBS (about 4° C), after which, the cells comprising the cell layer were fixed in 4% paraformaldehyde in PBS at ambient room temperature for 15 min. After fixation, the cells were permeabilized by 0.1% Triton X-100 in PBS for 10 min after which, the cells were  
5 rinsed by PBS for three times. The cells were incubated in 10 µM phalloidin/ 1 % (w/v) BSA solution for 20 min followed by PBS rinsing for 3 times. The cells were then incubated in 10 µM Topro-3 for 20 min, followed by PBS wash for 3 times. The film was set on a microscope slide and examined by CLSM. The blue color is attributed to nuclei stained by TO-PRO®-3. The green color is attributed to the F-actin in the cellular cytoskeleton stained by phalloidin.  
10 The red color is attributed to rhodamine-labelled proteins.

Example 5: Assessment of osteogenic differentiation of mouse BCMs in the cell layer of free-standing nanomembranes

A study was done to assess the effects of adding a cell regular into one of the layers comprising the LBL nanomembranes of the present disclosure, on affecting regulation of  
15 mammalian cells. For this study, bone morphogenic protein-2 (BMP-2) was used as a regulator to determine if adding BMP-2 to one of the layers of the present free-standing nanomembranes could regulate the osteogenic differentiation of BMSCs cells. BMSCs cells have the capacity to differentiate into a variety of cell types and have been widely used in tissue engineering. Bone morphogenetic proteins play a key role in osteogenic differentiation  
20 and bone development and can drive uncommitted mesenchymal precursor cells toward the osteoblast lineage.

Cell-gelatin-(chitosan/alginate)<sub>3</sub> nanomembranes comprising a cell layer of mouse BMSCs cells were fabricated following the method disclosed in Example 3. During the fabrication process, each layer of chitosan and each layer of alginate comprised 10 ng/ml of  
25 BMP-2 (Product No. CYT-261, ProSpec, New Brunswick, NJ, USA). These membranes were designated as “cell-gelatin- (chitosan / alginate)<sub>3</sub> -BMP2” nanomembranes. Also fabricated were nanomembranes comprising layers of tissue culture polystyrene (TCPS) following the LBL method taught by Kharlampieva et al. (2007, *Electrostatic Layer-by-Layer Self-Assembly of Poly(carboxybetaine): Role of Zwitterions in Film Growth*.  
30 Macromolecules 40(10):3663-3668), and TCPS nanomembranes having one layer provided with 10 ng/ml BMP-2 (TCPS+BMP2).

The three types of nanomembranes i.e., (i) TCPS (negative controls), (ii) TCPS+BMP2 (positive controls, and (iii) cell-gelatin- (chitosan / alginate)<sub>3</sub> -BMP2, were cultured at 37° C in DMEM supplemented with high glucose, 10% FBS, 100 unit/ml penicillin, and 100 ug/ml streptomycin.

Quantitative PCR was used to assess gene expression of the following osteoblast differentiation markers in the BMSCs cell layers in each of the three types of nanomembranes. Bone sialoprotein (BSP), runt-related transcription factor 2 (Runx2), collagen type I (COL-I), and osteopontin (OPN) are considered as lineage-specific markers of osteoblastic differentiation. BSP is a highly sulfated, phosphorylated, and glycosylated protein that mediates cell attachment. Runx2 is essential for osteoblastic differentiation and bone formation. COL-I is the major organic component of bone matrix produced by osteoblasts. It functions as a scaffold of mineralization in bone. OPN is a phosphoprotein member of the SIBLING family that possesses several calcium- binding domains and is associated with cell attachment, proliferation, and mineralization of extracellular matrix into bone, synthesized by bone-forming cells.

RNA was extracted from cell layers at days 3 and 7 using TRIZOL<sup>®</sup> reagent (TRIZOL is a registered trademark of Molecular Research Center Inc., Cincinnati, OH, USA). Starting from 1µg RNA, 20 µl cDNA were synthesized using a VERSO<sup>®</sup> cDNA kit (VERSO is a registered trademark of Advanced Biotechnologies Inc., Surrey, UK) with oligo-dT primer in the presence of dNTP. Then quantitative real-time PCR was performed by SYBER Green assays (GeneCopoeia Inc, USA). Amplification conditions were as follows: hold for 10 minutes at 95° C, followed by 40 cycles of 15-sec at 95° C and 1 min at 60° C. Thermal cycling and fluorescence detection were done using the STEPONEPLUS<sup>®</sup> Real-Time PCR System (STEPONEPLUS is a registered trademark of Applied Biosystems LLC, Foster City, CA, USA). The mRNA expression levels were determined relative to the GAPDH by the  $\Delta C_t$  method. The primer sequences used are shown in Table 1.

Table 1: Primer sequences used for qRT-PCR

| Gene* | SEQ ID NO: | Forward primer sequence        | SEQ ID NO: | Reverse primer sequence        |
|-------|------------|--------------------------------|------------|--------------------------------|
| BSP   | 1          | 5'-ccacactttccacactctcg-3'     | 2          | 5'-cgtcgctttccttcacttttg-3'    |
| Runx2 | 3          | 5'-gctattaaagtgacagtggacgg -3' | 4          | 5'-ggcgatcagagaacaaaactagg -3' |
| COL-I | 5          | 5'-aacagtcgcttcacctacag -3'    | 6          | 5'-aatgtccaaggaggagccac -3'    |
| OPN   | 7          | 5'-ctacgacctgagattggcag-3'     | 8          | 5'-catgtggctataggatctggg -3'   |
| GAPDH | 9          | 5'-aggtegggtgtaacggatttg-3'    | 10         | 5'-tgtagaccatgtagttgagggtca-3' |

\* BSP = bone sialoprotein

Runx2 = runt-related transcription factor 2

COL-I = collagen type I

5 OPN = osteopontin

GAPDH = glyceraldehyde-3-phosphate dehydrogenase

Figs. 4(A)-4(D) show that mRNA expression of Runx2, COL-I, BSP, and OPN after a 3-day incubation decreased significantly in the cell-gelatin-(chitosan/alginate)<sub>3</sub>-BMP2 nanomembrane compared to the TCPS+BMP2 nanomembrane. However, the expression levels of all four genes were significantly greater in the cell-gelatin-(chitosan/alginate)<sub>3</sub>-BMP2 compared to their expressions in the TCPS+BMP2 nanomembranes and in the TCPS nanomembranes. These results suggest that osteoblast differentiation of mouse BMSCs cells can be affected, i.e. modulated by incorporation of cell regulators into one or more of the polyelectrolyte layers comprising the gelatin-(chitosan/alginate)<sub>x</sub> nanomembranes of the present disclosure.

## CLAIMS

1. A method for preparing a layered mammalian-cell compatible nanomembrane, comprising the steps of:

- (1) providing a support substrate having cleaned surfaces;
- (2) grafting a temperature-responsive substrate onto the support substrate;
- (3) culturing a layer of selected mammalian cells onto the surface of the temperature-responsive substrate;
- (4) depositing a layer of a gelatin solution onto the layer of mammalian cells;
- (5) depositing a first layer of a cationic polyelectrolyte solution onto the layer of gelatin;
- (6) depositing a first layer of an anionic polyelectrolyte solution onto the layer of cationic polyelectrolyte; and
- (7) storing the layered mammalian-cell compatible nanomembrane in a fluid medium at a first temperature wherein the cell layer remains attached to the temperature-responsive substrate.

2. The method of claim 1, additionally comprising adjusting the temperature of the fluid medium to a second temperature wherein the cell layer becomes detached from the temperature-responsive substrate.

3. The method of claim 2, additionally comprising storing the detached layered mammalian cell-compatible nanomembrane in a fresh fluid medium.

4. The method of claim 1, wherein the support substrate is a glass article.

5. The method of claim 1, wherein the temperature-responsive substrate is poly(N-isopropylacrylamide) and is grafted onto the support substrate by irradiation with a 365-nm UV light source.

6. The method of claim 1, wherein the gelatin solution comprises about 0.01% w/v to about 0.5% w/v of a type A gelatin.

7. The method of claim 1, wherein the cationic polyelectrolyte solution comprises about 0.01% w/v to about 0.5% w/v of a chitosan.

8. The method of claim 1, wherein the cationic polyelectrolyte solution comprises about 5 ng/ml to about 200 ng/ml of a cellular regulatory compound selected from a group consisting of bone morphogenetic proteins, dexamethasone, ascorbic acid,  $\beta$ -glycerol phosphate, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), tissue inhibitor of metalloproteinase (TIMP), neuropilin-1 (NRP-1), and combinations thereof.

9. The method of claim 1, wherein the cationic polyelectrolyte solution comprises about 0.001% w/w to about 10% w/w of an antibiotic selected from a group consisting of aminoglycosides, azoles,  $\beta$ -lactam antibiotics, cephalosporins, chloramphenicol, clindamycin, fusidic acid, glycopeptides, macrolides, metronidazole, mupirocin, penicillins, polyenes, quinolones, rifamycins, sulfonamides, tetracyclines, trimethoprim, and combinations thereof among others.

10. The method of claim 1, wherein the cationic polyelectrolyte solution comprises about 0.001% w/w to about 0.1% w/w of micronutrients selected from a group consisting of Vitamin A, the Vitamin B group, Vitamin C, Vitamin D, Vitamin E, Vitamin K, carotinoids, and combinations thereof.

11. The method of claim 1, wherein the anionic polyelectrolyte solution comprises about 0.01% w/v to about 0.5% w/v of an alginate.

12. The method of claim 1, wherein the anionic polyelectrolyte solution comprises about 5 ng/ml to about 200 ng/ml of a cellular regulatory compound selected from a group consisting of bone morphogenetic proteins, dexamethasone, ascorbic acid,  $\beta$ -glycerol phosphate, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), tissue inhibitor of metalloproteinase (TIMP), neuropilin-1 (NRP-1), and combinations thereof.

13. The method of claim 1, wherein the anionic polyelectrolyte solution comprises about 0.001% w/w to about 10% w/w of an antibiotic selected from a group consisting of

aminoglycosides, azoles,  $\beta$ -lactam antibiotics, cephalosporins, chloramphenicol, clindamycin, fusidic acid, glycopeptides, macrolides, metronidazole, mupirocin, penicillins, polyenes, quinolones, rifamycins, sulfonamides, tetracyclines, trimethoprim, and combinations thereof among others.

14. The method of claim 1, wherein the anionic polyelectrolyte solution comprises about 0.001% w/w to about 0.1% w/w of micronutrients selected from a group consisting of Vitamin A, the Vitamin B group, Vitamin C, Vitamin D, Vitamin E, Vitamin K, carotinoids, and combinations thereof.

15. The method of claim 1, wherein the fluid medium is a Dulbecco's modified Eagle's medium.

16. The method of claim 1, additionally comprising a layer of an anionic polyelectrolyte solution onto the gelatin layer before depositing the cationic polyelectrolyte solution.

17. The method of claim 1, additionally comprising sequentially applying a second layer of the cationic polyelectrolyte solution and a second layer of the anionic polyelectrolyte solution.

18. The method of claim 17, wherein steps (4)-(6) are repeated at least one additional time.

19. A layered mammalian-cell compatible nanomembrane prepared according to the method of claim 1.

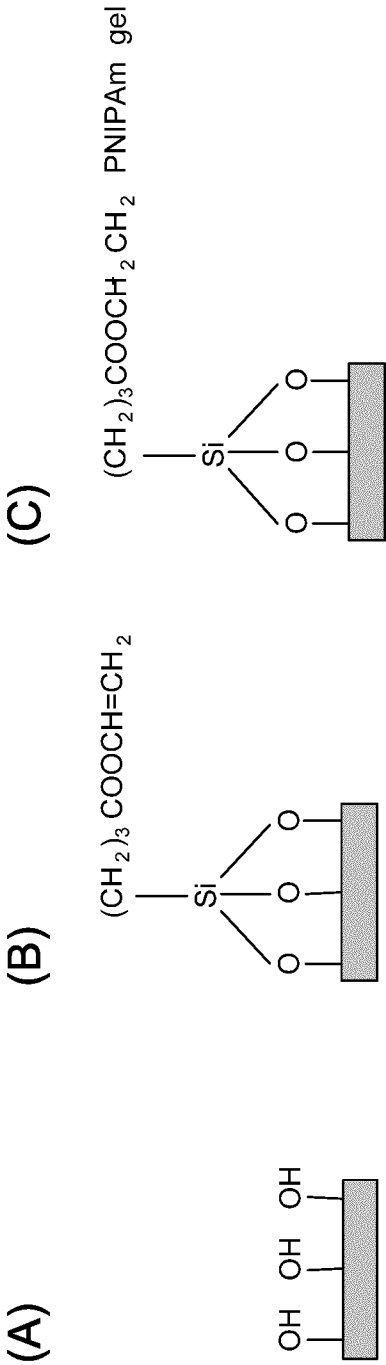


Fig. 1

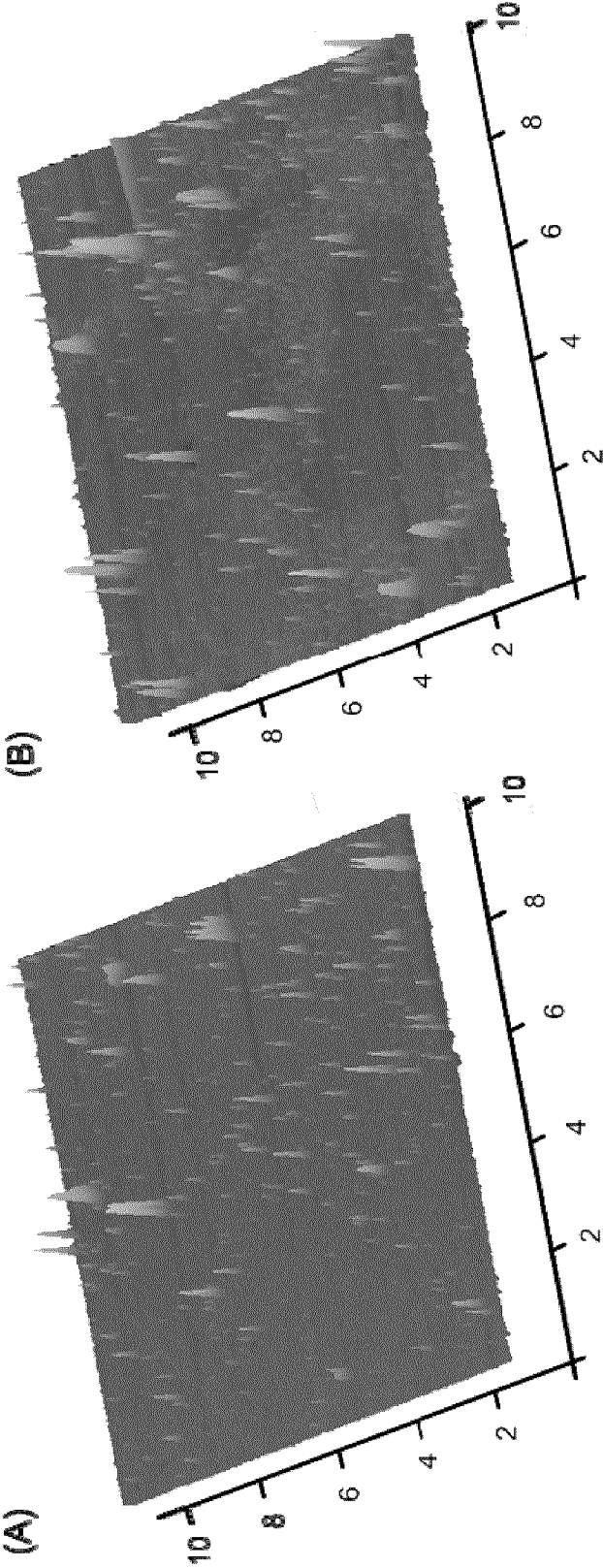


Fig. 2

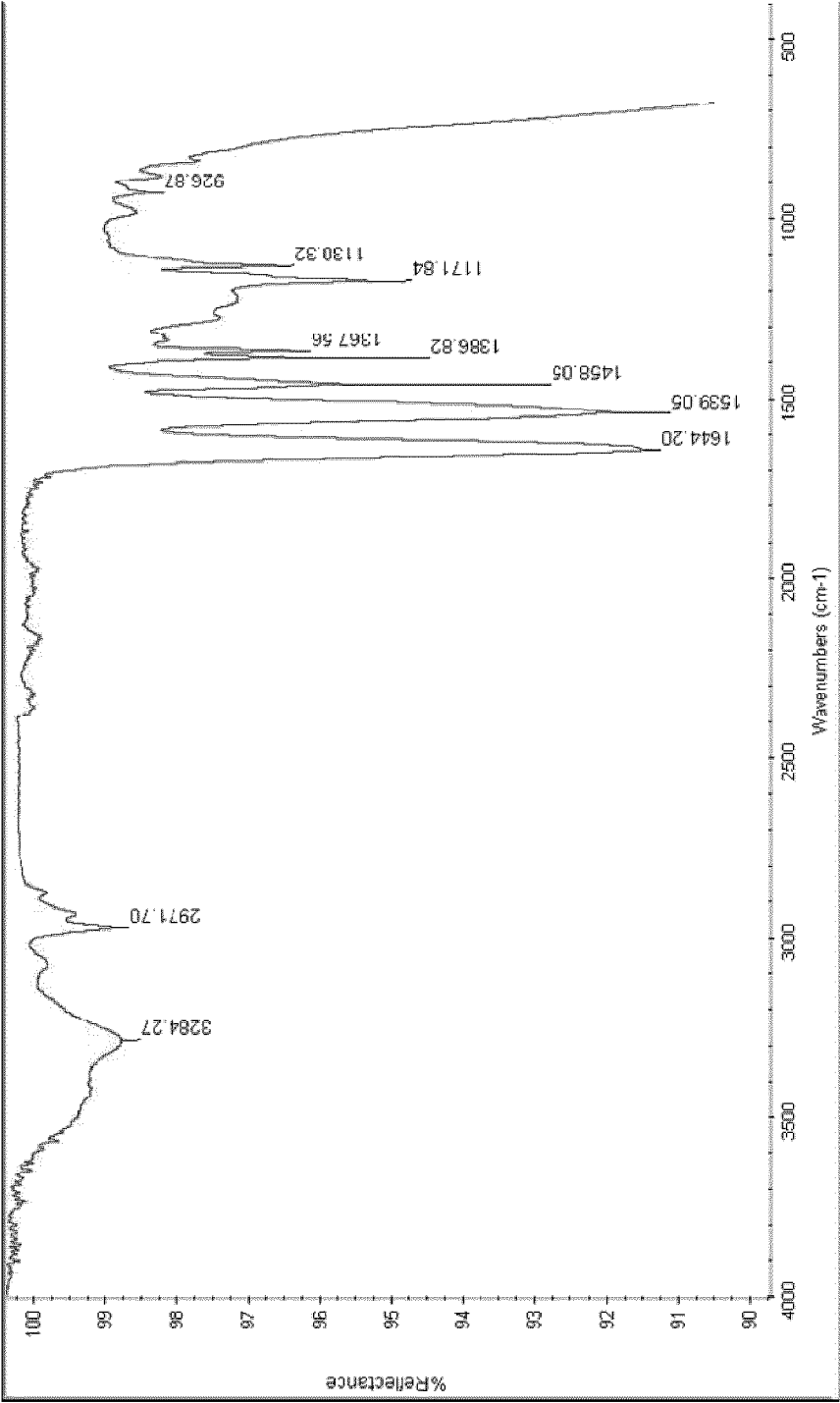


Fig. 3

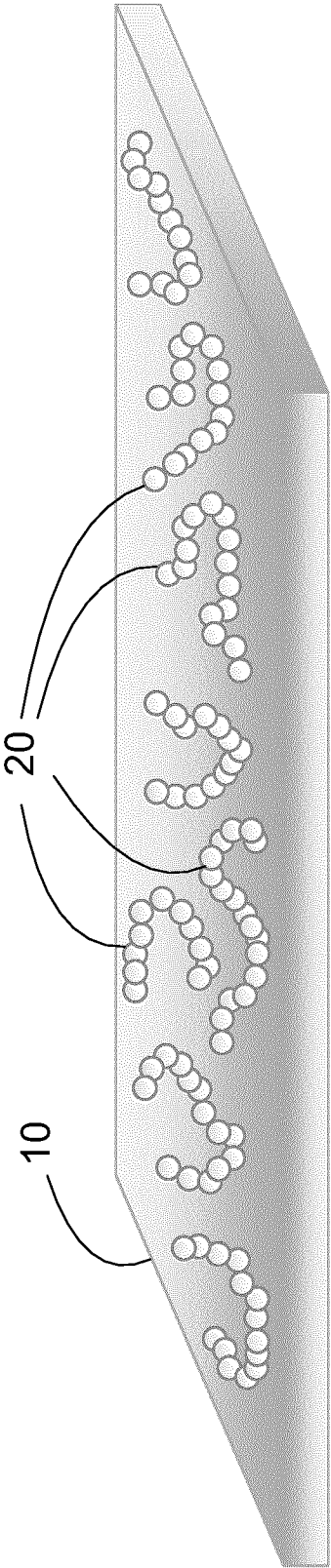


Fig. 4

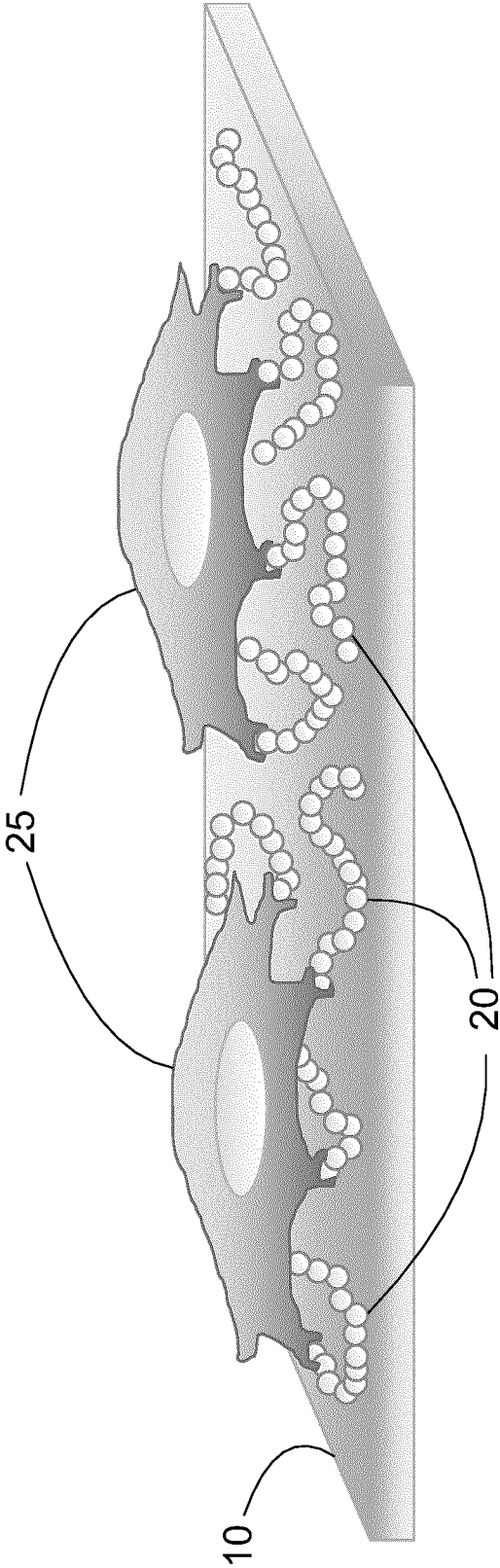


Fig. 5

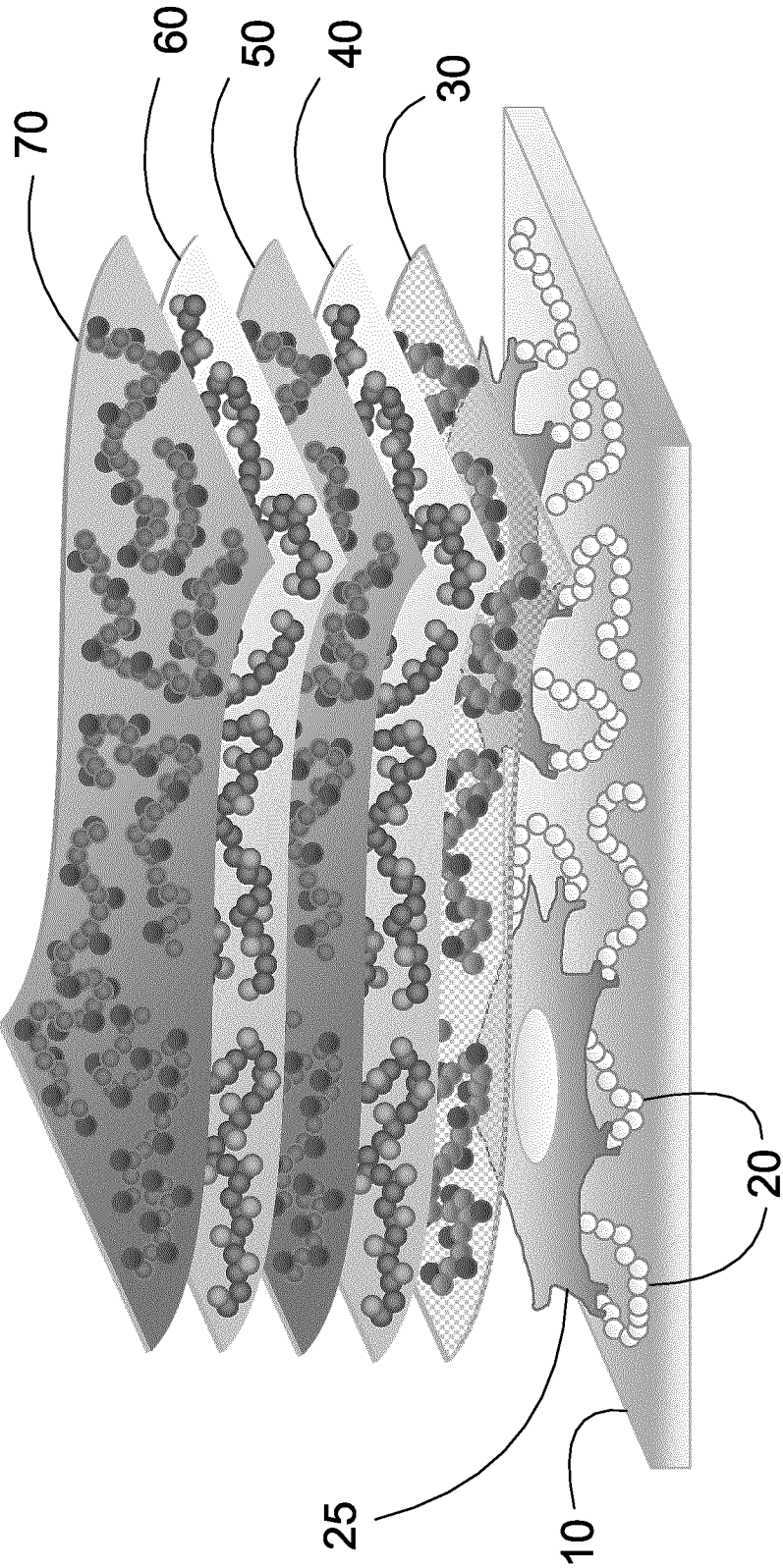


Fig. 6

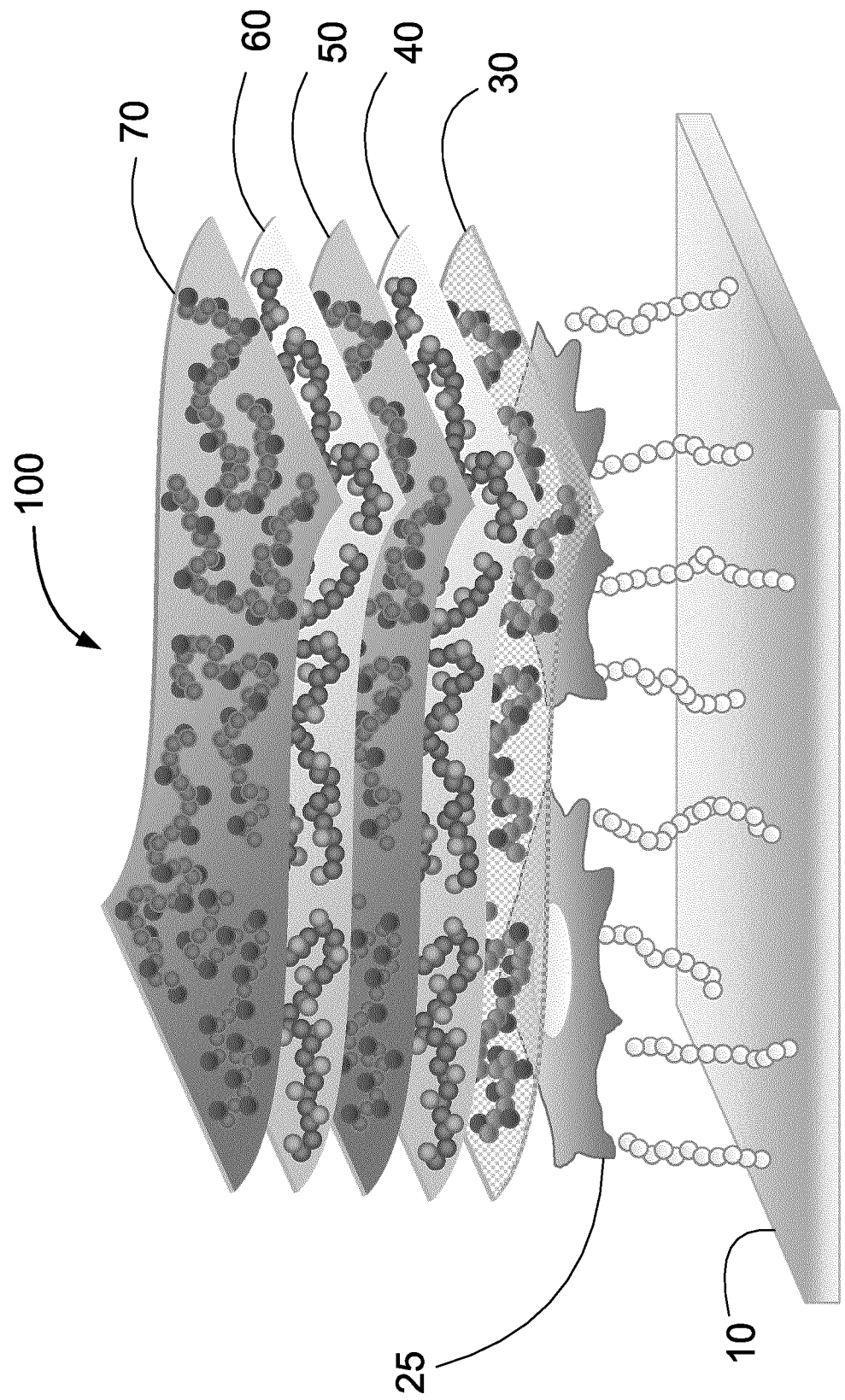


Fig. 7

8/17



Fig. 8

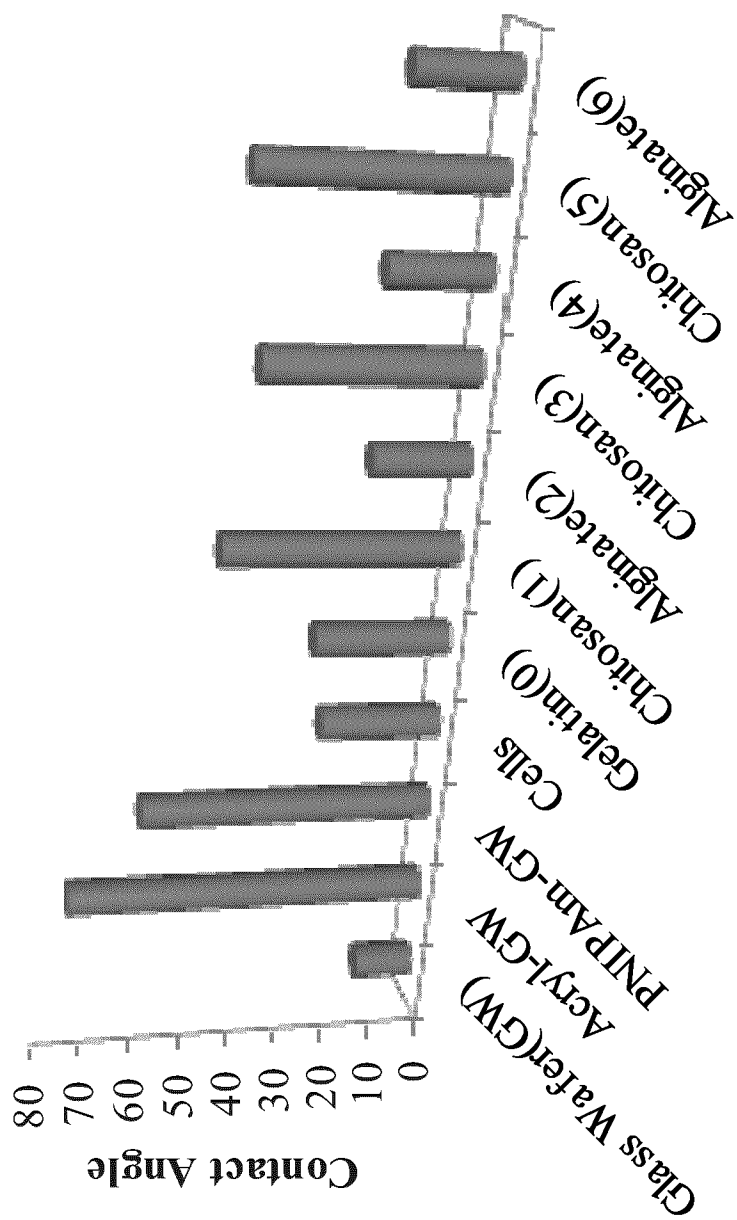


Fig. 9

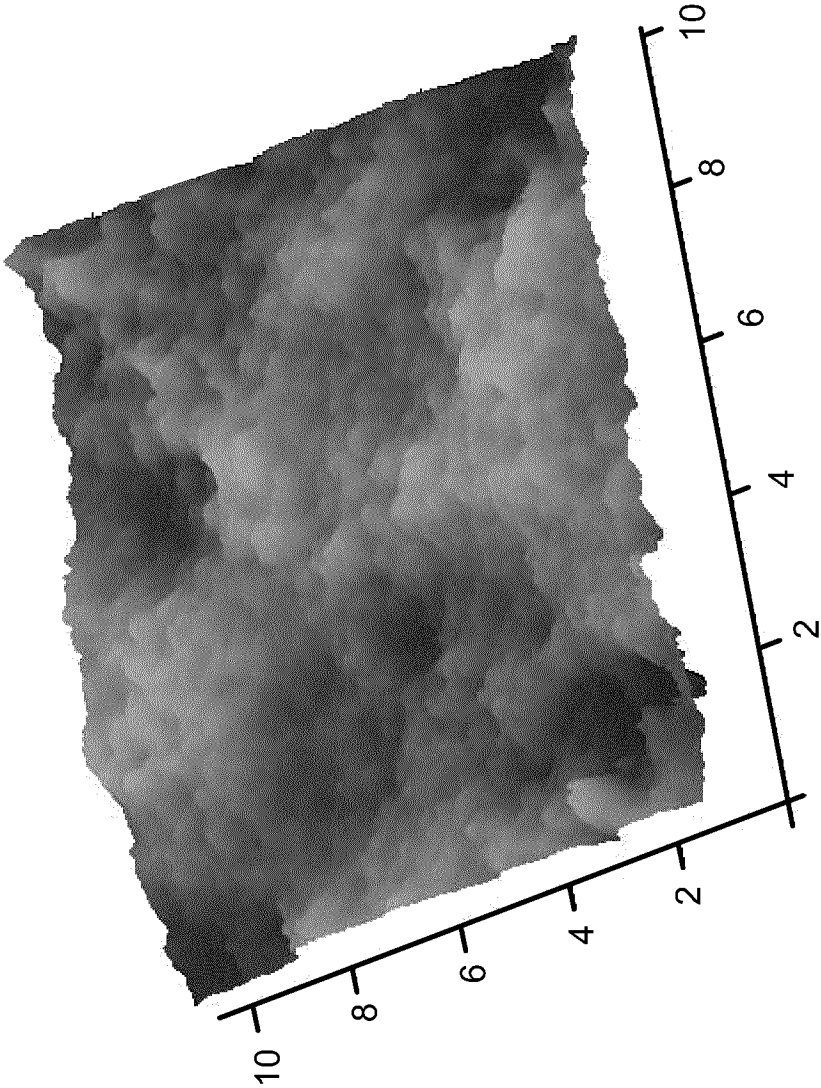


Fig. 10

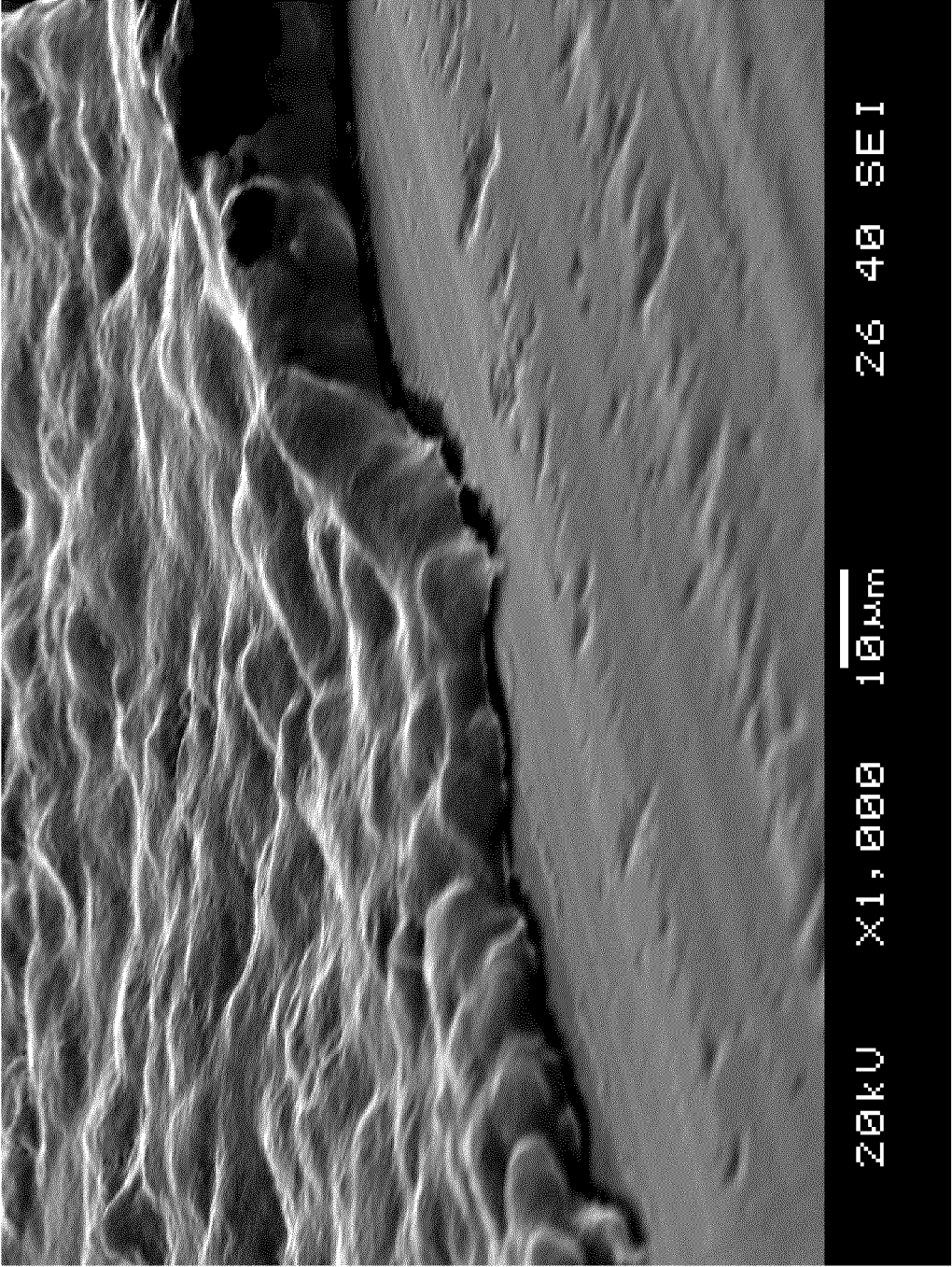


Fig. 11

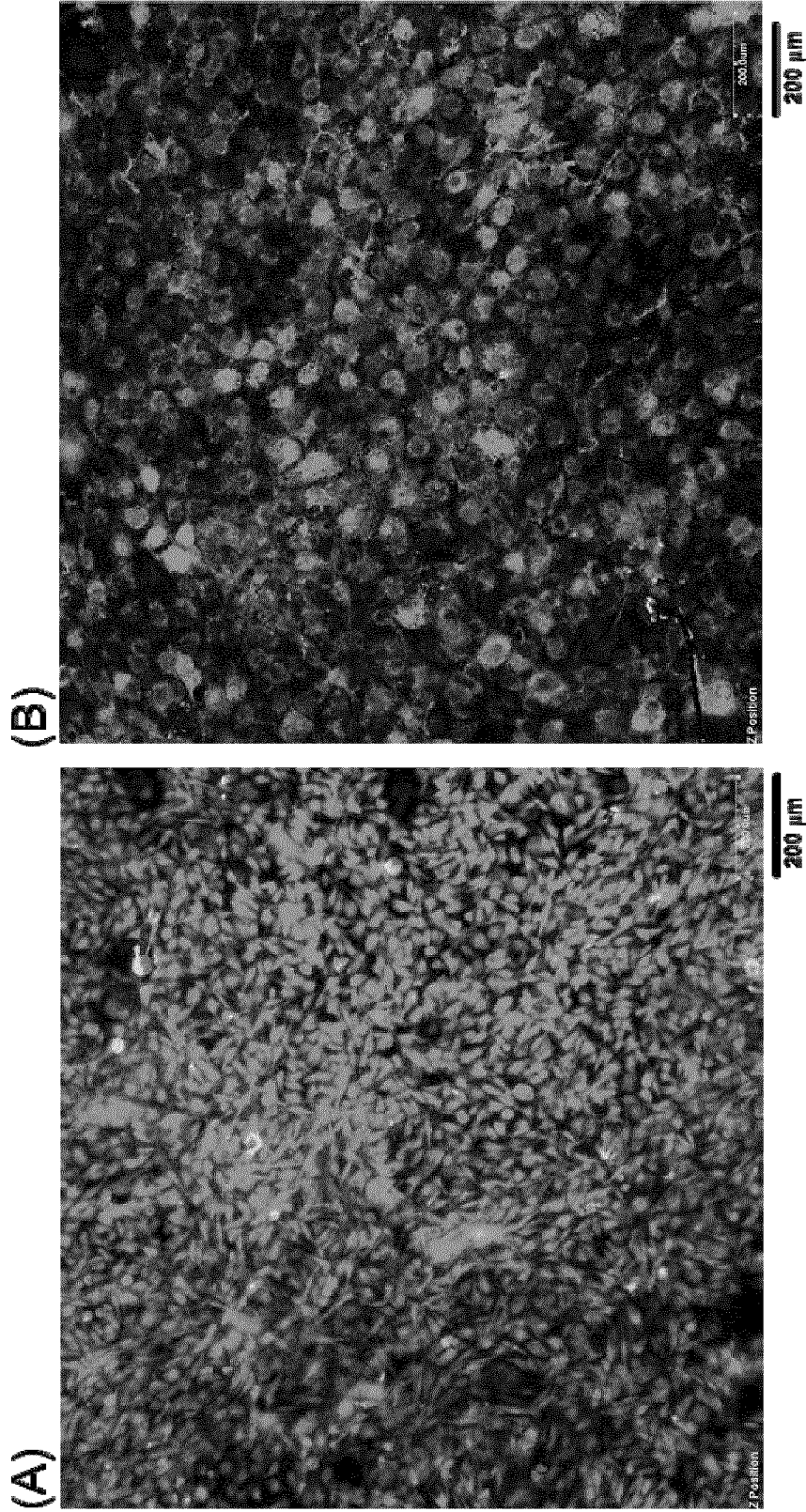
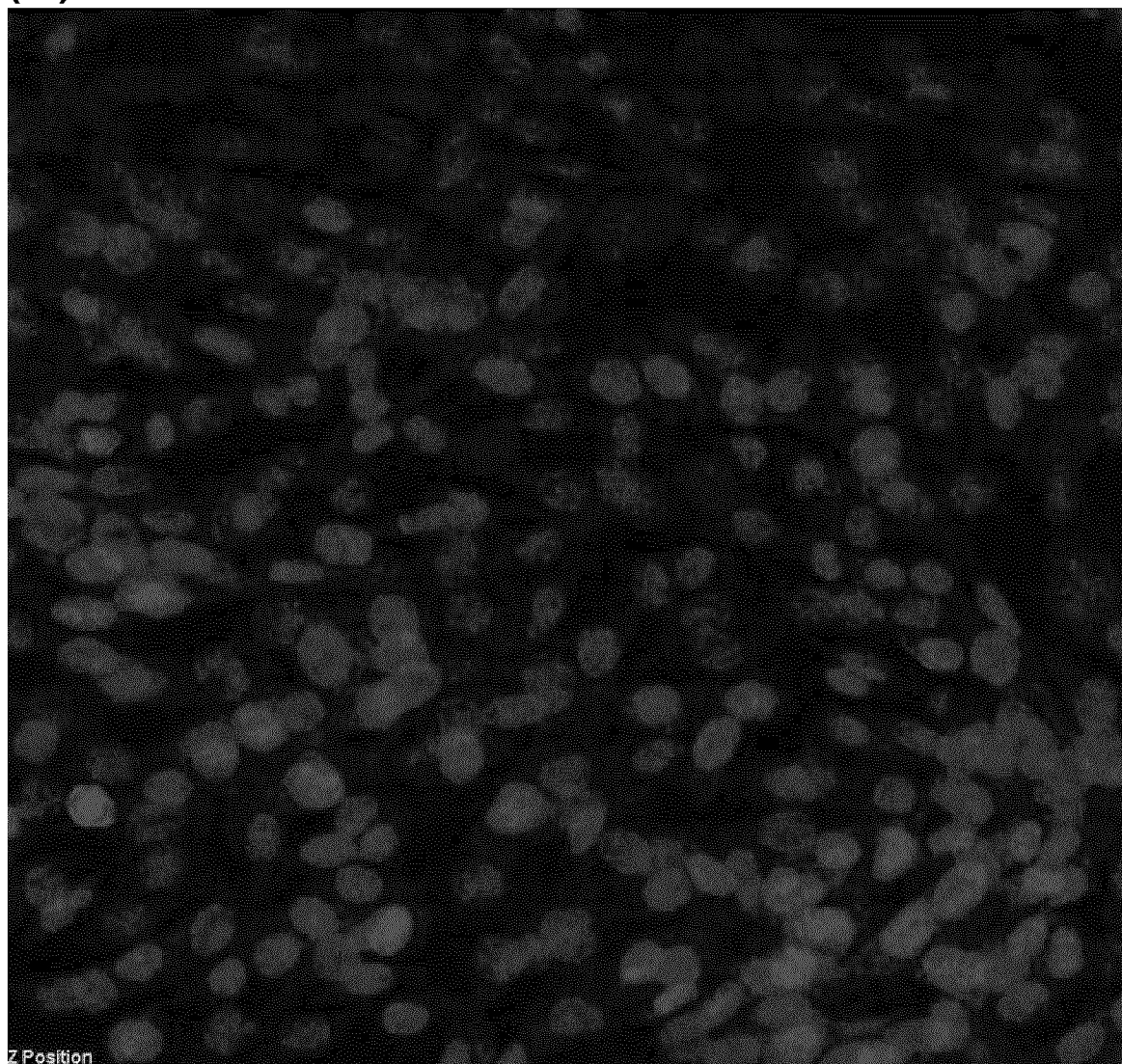


Fig. 12

(A)

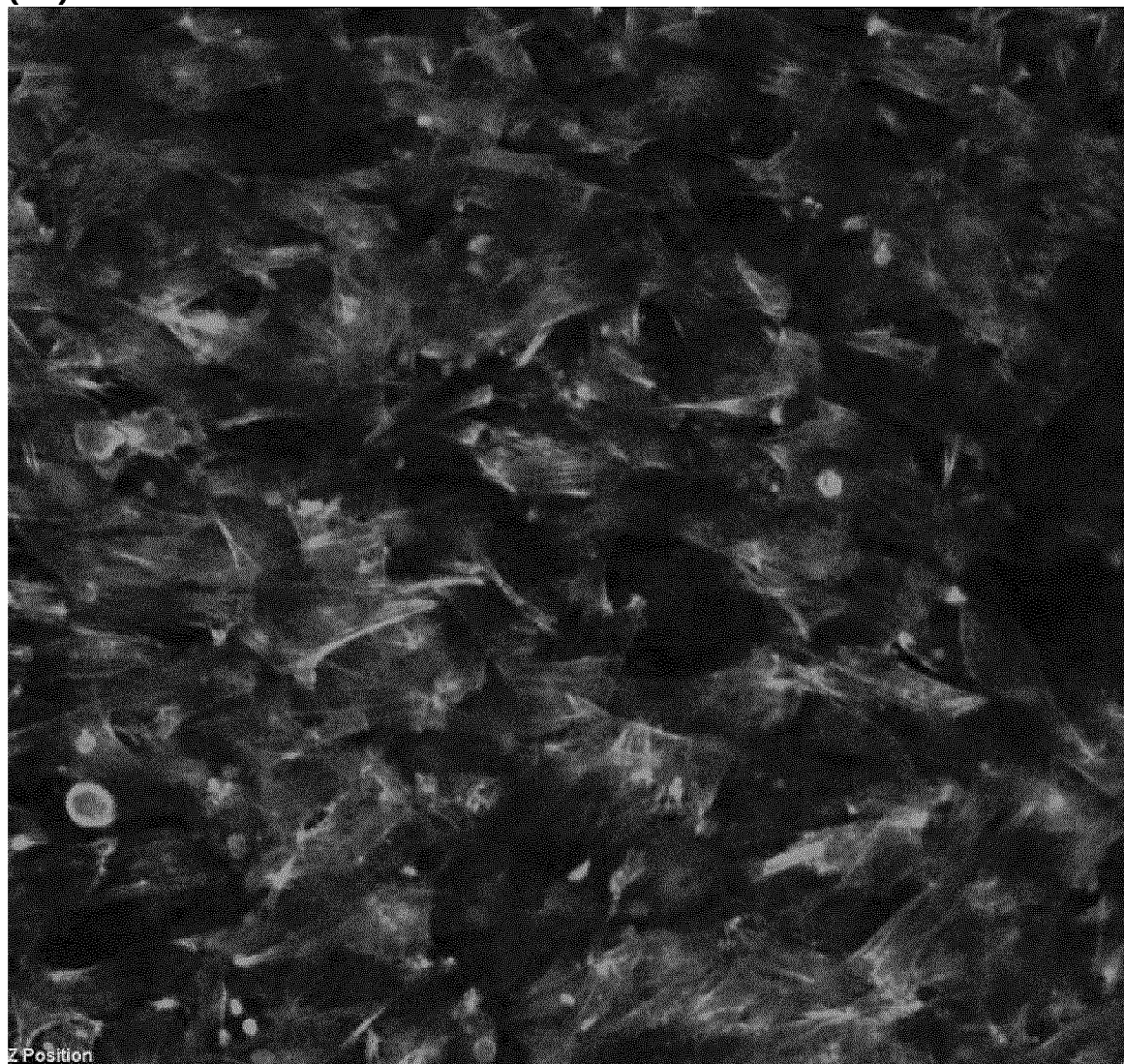


(B)



Fig. 13

(A)



(B)

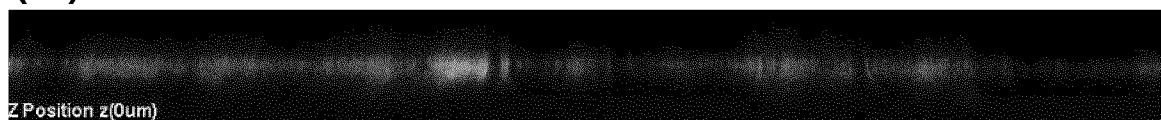
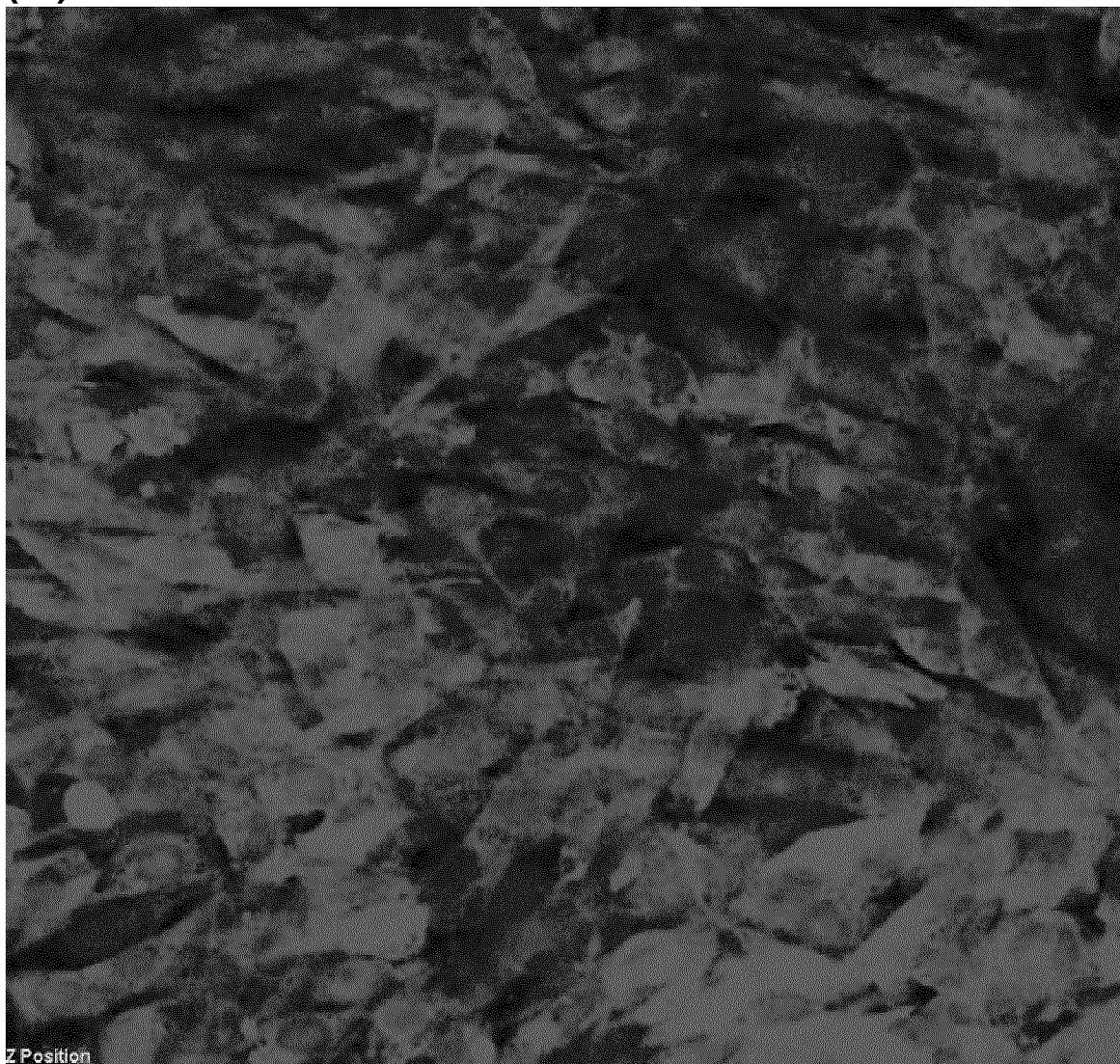


Fig. 14

(A)



(B)

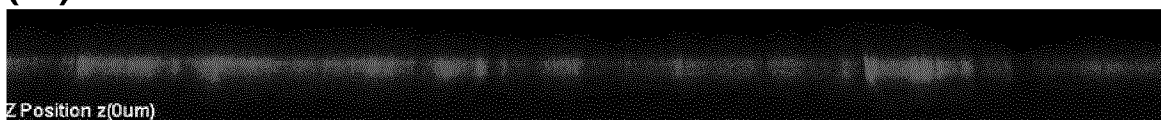
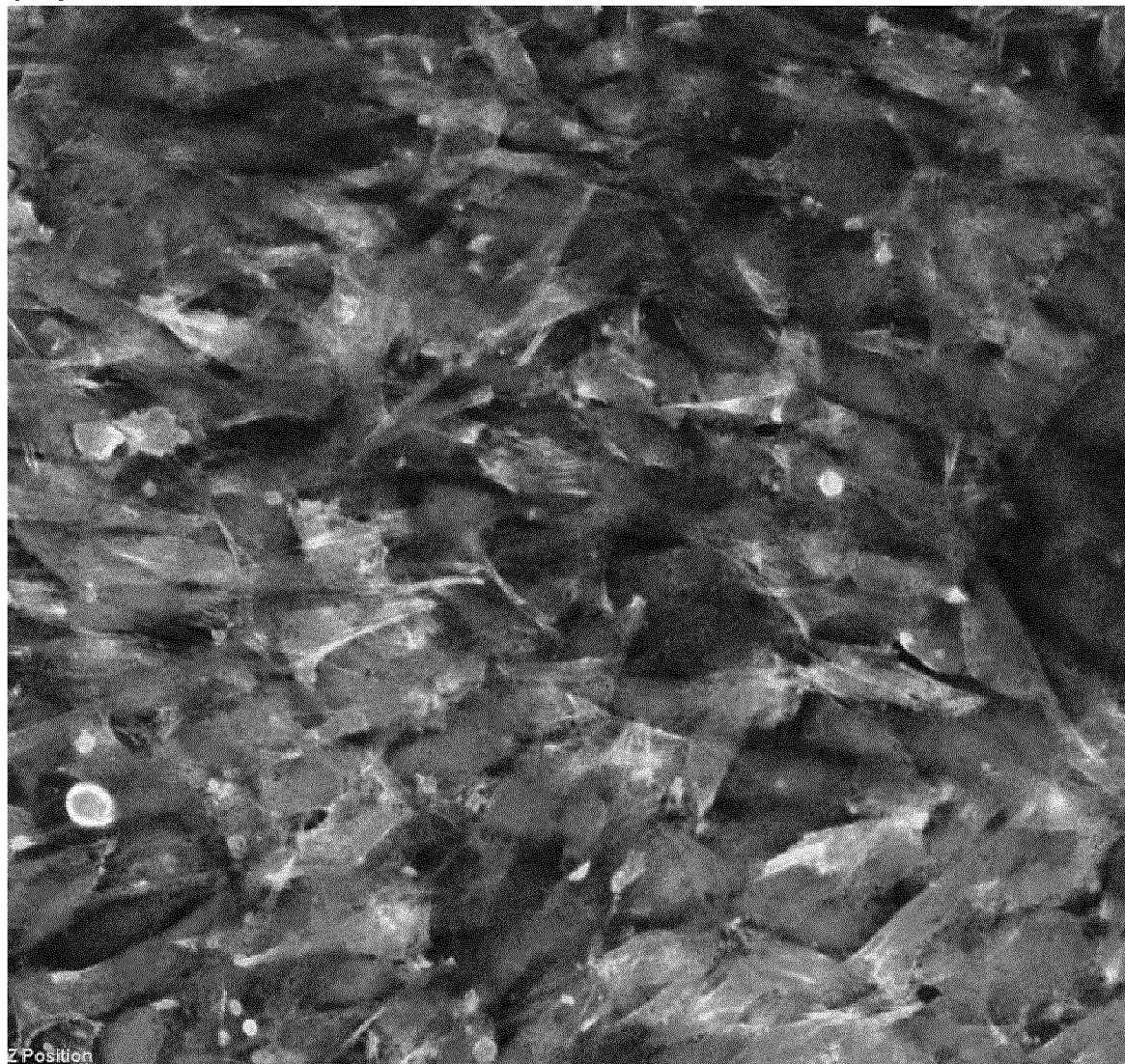


Fig. 15

(A)



(B)

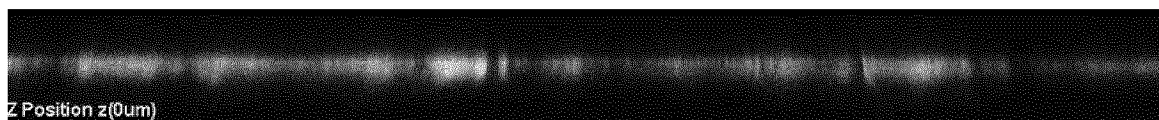


Fig. 16

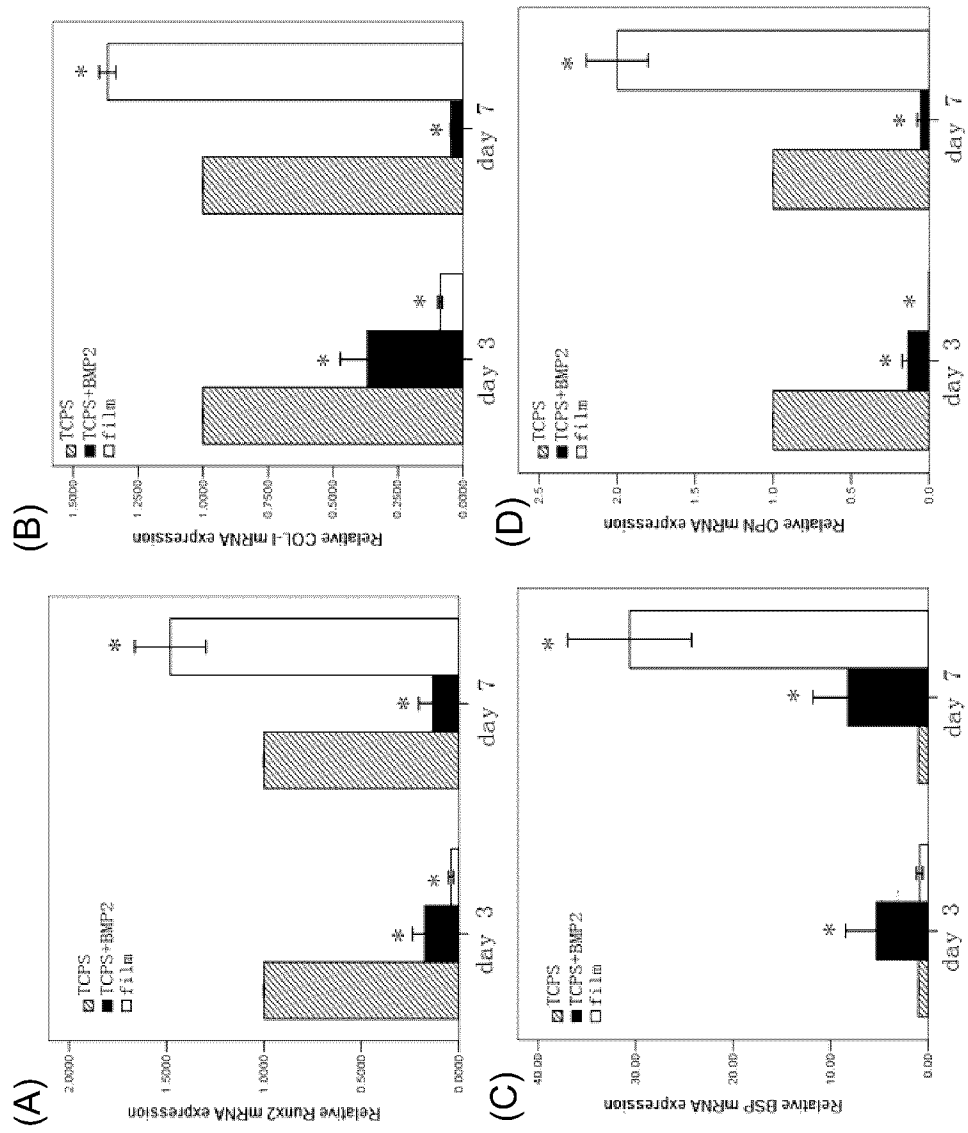


Fig. 17

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/CA2013/050872

| <p>A. CLASSIFICATION OF SUBJECT MATTER</p> <p>IPC: <i>C12N 11/04</i> (2006.01), <i>A61L 27/38</i> (2006.01), <i>C12N 11/02</i> (2006.01), <i>C12N 5/071</i> (2010.01), <i>C12M 3/04</i> (2006.01)</p> <p>According to International Patent Classification (IPC) or to both national classification and IPC</p>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |                                                                                                                                                                                                                                                  |                                                                                               |                                           |                                                                                                                                                                                                     |                                                                                                          |                                                                                                                                                                              |                                                                                                                                                                             |                                                                                                                                                                                                                                                  |                                                                                                                                                                         |                                                                                 |                                                                              |   |                                                                                                        |    |
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| <p>B. FIELDS SEARCHED</p> <p>Minimum documentation searched (classification system followed by classification symbols)</p> <p>IPC: <i>C12N 11/04</i> (2006.01), <i>A61L 27/38</i> (2006.01), <i>C12N 11/02</i> (2006.01), <i>C12N 5/071</i> (2010.01), <i>C12M 3/04</i> (2006.01)</p> <p>Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched</p> <p>IPC: C12N 11</p> <p>Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)</p> <p>Databases: Total Patent, PubMed, Canadian Patent Database; Keywords: pnipam, temperature-responsive, polyelectrolyte, anionic, cationic, alginate, chitosan, gelatin</p>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |                                                                                                                                                                                                                                                  |                                                                                               |                                           |                                                                                                                                                                                                     |                                                                                                          |                                                                                                                                                                              |                                                                                                                                                                             |                                                                                                                                                                                                                                                  |                                                                                                                                                                         |                                                                                 |                                                                              |   |                                                                                                        |    |
| <p>C. DOCUMENTS CONSIDERED TO BE RELEVANT</p> <table border="1"> <thead> <tr> <th>Category*</th> <th>Citation of document, with indication, where appropriate, of the relevant passages</th> <th>Relevant to claim No.</th> </tr> </thead> <tbody> <tr> <td>X</td> <td>US 2005/0233442 A1 (TODA, S) 20 October 2005 (20-10-2005)<br/>* the whole document, particularly, paragraphs [0012], [0013], [0022], [0031], [0034], [0054], and Example 4 *</td> <td>19</td> </tr> <tr> <td>X</td> <td>JP2007215519 A (TODA, S) 30 August 2007 (30-08-2007)<br/>* Machine translation *</td> <td>19</td> </tr> <tr> <td>X</td> <td>JP2007259776 A (TODA, S) 11 October 2007 (11-10-2007)<br/>* Machine translation *</td> <td>19</td> </tr> </tbody> </table>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |                                                                                                                                                                                                                                                  |                                                                                               | Category*                                 | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                  | Relevant to claim No.                                                                                    | X                                                                                                                                                                            | US 2005/0233442 A1 (TODA, S) 20 October 2005 (20-10-2005)<br>* the whole document, particularly, paragraphs [0012], [0013], [0022], [0031], [0034], [0054], and Example 4 * | 19                                                                                                                                                                                                                                               | X                                                                                                                                                                       | JP2007215519 A (TODA, S) 30 August 2007 (30-08-2007)<br>* Machine translation * | 19                                                                           | X | JP2007259776 A (TODA, S) 11 October 2007 (11-10-2007)<br>* Machine translation *                       | 19 |
| Category*                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                               | Relevant to claim No.                                                                         |                                           |                                                                                                                                                                                                     |                                                                                                          |                                                                                                                                                                              |                                                                                                                                                                             |                                                                                                                                                                                                                                                  |                                                                                                                                                                         |                                                                                 |                                                                              |   |                                                                                                        |    |
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| <p><input type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.</p> <table border="1"> <tbody> <tr> <td>* Special categories of cited documents :</td> <td>"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</td> </tr> <tr> <td>"A" document defining the general state of the art which is not considered to be of particular relevance</td> <td>"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</td> </tr> <tr> <td>"E" earlier application or patent but published on or after the international filing date</td> <td>"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</td> </tr> <tr> <td>"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)</td> <td>"&amp;" document member of the same patent family</td> </tr> <tr> <td>"O" document referring to an oral disclosure, use, exhibition or other means</td> <td></td> </tr> <tr> <td>"P" document published prior to the international filing date but later than the priority date claimed</td> <td></td> </tr> </tbody> </table> |                                                                                                                                                                                                                                                  |                                                                                               | * Special categories of cited documents : | "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 | "A" document defining the general state of the art which is not considered to be of particular relevance | "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 | "E" earlier application or patent but published on or after the international filing date                                                                                   | "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 | "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) | "&" document member of the same patent family                                   | "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 |    |
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| <p>Date of the actual completion of the international search</p> <p>09 January 2014 (09-01-2014)</p>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |                                                                                                                                                                                                                                                  | <p>Date of mailing of the international search report</p> <p>30 January 2014 (30-01-2014)</p> |                                           |                                                                                                                                                                                                     |                                                                                                          |                                                                                                                                                                              |                                                                                                                                                                             |                                                                                                                                                                                                                                                  |                                                                                                                                                                         |                                                                                 |                                                                              |   |                                                                                                        |    |
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**INTERNATIONAL SEARCH REPORT**  
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International application No.  
**PCT/CA2013/050872**

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