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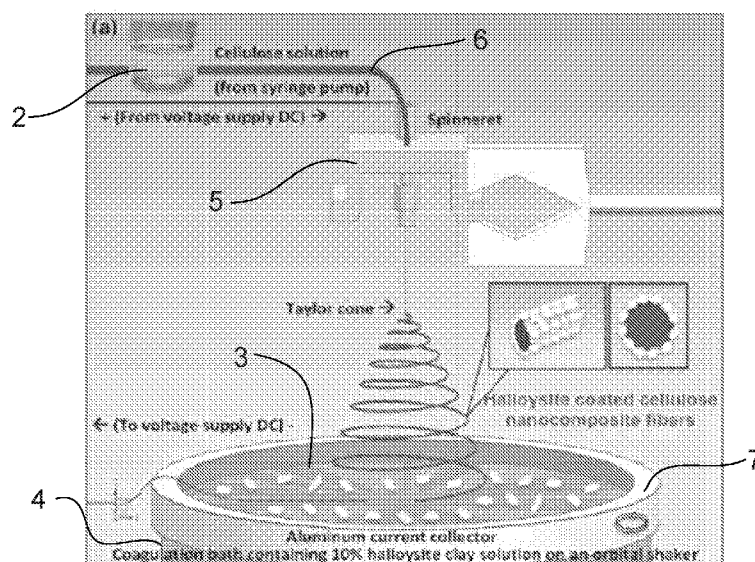


FIG. 3A

(57) Abstract: A method of making a cellulose - nanoclay hemostatic nanocomposite fiber, including the steps of preparing a homogeneous cellulose solution including cellulose and a room temperature ionic liquid, preparing a nanoclay suspension including halloysite and distilled water, electrospinning the cellulose solution into a first bath including the nanoclay suspension, transferring solidified cellulose - halloysite fibers from the first bath to a second bath including ethanol and distilled water, removing the solidified cellulose - halloysite fibers from the second bath, and freeze-drying the solidified cellulose - halloysite fibers.



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NANOCOMPOSITE FIBERS WITH A DRAMATIC REDUCTION IN HUMAN PLASMA COAGULATION TIME

CROSS REFERENCE TO RELATED APPLICATION(S)

[1] This application claims the benefit of U.S. Provisional Patent Application No. 62/799,090, filed January 31, 2019, which is incorporated by reference as if disclosed herein in its entirety.

FIELD

[2] The present technology relates to nanocomposite fibers and, more particularly, to methods of forming nanocomposite fibers having improved hemostatic qualities.

BACKGROUND

[3] There remains an urgent need in the medical community for the development of hemostatic agents to treat hemorrhage associated with traumatic injuries, surgical procedures, and frequent bleeding events resulting from blood coagulation disorders. Patients suffering from hereditary blood clotting disorders, such as hemophilia and von Willebrand disease, suffer from often unexplained and recurrent internal and external bleeding episodes throughout their lives. These external bleeding episodes include prolonged nosebleeds (epistaxis), bleeding from the gums, and excessive bleeding from small cuts or wounds. Even though many of these episodes are non-life-threatening, they can cause extreme discomfort in such patients. In the case of severe hemorrhage, it is critical to cease bleeding as quickly as possible to prevent imminent death. On a battlefield, most fatalities are due to severe hemorrhage and transpire within the first hour of receiving a wound. Thus, the patient's chance of survival depends on a first responder's ability to minimize blood loss.

[4] There have been a number of topical anti-hemorrhagic agents developed to stop external hemorrhages. Many consist of a polymer or polymer blend mixed with a blood-clotting agent. HemCon, Chitoseal, and Celos represent several commercially available wound dressings that contain the biopolymer chitosan as the hemostatic agent. Most common commercially available hemostatic dressings, including Quikclot Combat Gauze® ("QCG"), are produced either by immersing or spraying the fibrous substrate with a slurry or suspension containing a clotting agent. These products fail to provide prolonged clotting

activity because of the loss of clotting agent due to leaching. Extrusion of blends of polymers and clotting agents has also been used to produce hemostatic fibrous materials. However, the clotting agents inside these fibers have minimum or no contact with the external environment making these ineffective in promoting coagulation. Furthermore, the heterogeneous nature of extrusion processes negatively impacts the mechanical properties of these polymeric fiber products.

[5] Naturally occurring aluminosilicate clay is the most popular choice of hemostatic agent used in a wide range of hemostatic wound dressings. Kaolin aluminosilicate clay promotes coagulation by activating factor XII of the coagulation cascade without an accompanying exothermic reaction. In contrast, zeolite clays release thermal energy on contact with the water in blood. This exothermic reaction can cause second-degree burns in patients treated with zeolite-based hemostatic products. Thus, the current generation of clay-based topical hemostatic wound dressings almost exclusively contains kaolin clay. However, kaolin-based hemostatic products quickly lose effectiveness as the water in blood washes away, or leaches, the clotting agent. Therefore, an improved hemostatic agent is needed.

SUMMARY

[6] Accordingly, some embodiments of the present technology are directed to a halloysite nanoclay physically embedded onto the surface of cellulose nanofibers to prevent leaching of the clotting factors and to promote human plasma coagulation. In some embodiments, a halloysite clay hemostatic nanocomposite was fabricated using a one-step wet-wet electrospinning technique and evaluated for human plasma coagulation speed.

[7] According to a first embodiment of the present technology, a method of making a cellulose – nanoclay hemostatic nanocomposite fiber is provided. The method includes the steps of preparing a cellulose solution including cellulose and a non-volatile solvent, preparing a nanoclay suspension including an aluminosilicate and water, electrospinning the cellulose solution into a first bath including the nanoclay suspension, and removing solidified cellulose – nanoclay fibers from the first bath.

[8] In some embodiments, the non-volatile solvent is a room temperature ionic liquid.

- [9] In some embodiments, the cellulose solution is homogenous.
- [10] In some embodiments, the aluminosilicate is halloysite.
- [11] In some embodiments, the nanoclay suspension further includes alcohol.
- [12] In some embodiments, the method further includes freeze-drying the solidified cellulose – nanoclay fibers.
- [13] In some embodiments, the method further includes transferring the solidified cellulose – nanoclay fibers to a second bath including alcohol and water. In other embodiments, the method further includes removing the solidified cellulose nanoclay fibers from the second bath, and freeze-drying the solidified cellulose – nanoclay fibers.
- [14] In some embodiments, the solidified cellulose – nanoclay fibers have an average diameter of about 190 nm to about 710 nm.
- [15] In some embodiments, the solidified cellulose – nanoclay fibers are configured to coagulate human plasma in an average time of about 124 seconds to about 162 seconds.
- [16] According to another embodiment of the present technology, a method of making a cellulose – nanoclay hemostatic nanocomposite fiber is provided. The method including the steps of preparing a homogenous cellulose solution including cellulose and a room temperature ionic liquid, preparing a nanoclay suspension including halloysite and distilled water, electrospinning the cellulose solution into a first bath including the nanoclay suspension, transferring solidified cellulose – halloysite fibers from the first bath to a second bath including ethanol and distilled water, removing the solidified cellulose – halloysite fibers from the second bath, and freeze-drying the solidified cellulose – halloysite fibers.
- [17] In some embodiments, the solidified cellulose – halloysite fibers have an average diameter of about 450 nm.
- [18] In some embodiments, the solidified cellulose – halloysite fibers have a specific surface area of about $33.6 \text{ m}^2\text{g}^{-1}$.
- [19] In some embodiments, the solidified cellulose – halloysite fibers are configured to coagulate human plasma in an average time of about 143 seconds.

[20] In some embodiments, a cellulose – nanoclay hemostatic nanocomposite fiber made by the methods according to the embodiments discussed above is provided, wherein the nanoclay is halloysite.

[21] According to yet another embodiment of the present technology, an anti-hemorrhagic agent is provided. The anti-hemorrhagic agent includes a matrix of cellulose nanofibers, and a plurality of halloysite nanoclay particles attached to the matrix of cellulose nanofibers. The anti-hemorrhagic agent is configured to coagulate human plasma in an average time of about 143 seconds.

[22] In some embodiments, the plurality of halloysite nanoclay particles are attached to the matrix of cellulose nanofibers via a wet-wet electrospinning process. In some embodiments, the wet-wet electrospinning process includes the steps of preparing a homogenous cellulose solution including cellulose and a room temperature ionic liquid, preparing a nanoclay suspension including halloysite and distilled water, electrospinning the cellulose solution into a first bath including the nanoclay suspension, transferring solidified cellulose – halloysite fibers from the first bath to a second bath including ethanol and distilled water, removing the solidified cellulose – halloysite fibers from the second bath, and freeze-drying the solidified cellulose – halloysite fibers.

[23] In some embodiments, the anti-hemorrhagic agent further includes an article for applying the anti-hemorrhagic agent to a hemorrhage of a human user.

[24] According to an alternative embodiment of the present technology, a system of coagulating human plasma is provided. The system includes an article having a cellulose – halloysite hemostatic nanocomposite fiber, and a human user having a hemorrhage. The article is configured to apply the cellulose – halloysite hemostatic nanocomposite fiber to the hemorrhage.

[25] In some embodiments, the cellulose – halloysite hemostatic nanocomposite fiber is made by a wet-wet electrospinning process. In some embodiments, the wet-wet electrospinning process includes the steps of preparing a homogenous cellulose solution including cellulose and a room temperature ionic liquid, preparing a nanoclay suspension including halloysite and distilled water, electrospinning the cellulose solution into a first bath including the nanoclay suspension, transferring solidified cellulose – halloysite fibers from the first bath to a second bath including ethanol and distilled water, removing the solidified

cellulose – halloysite fibers from the second bath, and freeze-drying the solidified cellulose – halloysite fibers.

[26] In some embodiments, the cellulose – halloysite hemostatic nanocomposite fiber is configured to coagulate human plasma in an average time of about 143 seconds.

BRIEF DESCRIPTION OF THE DRAWINGS

[27] FIG. 1 is an isometric view of halloysite coated cellulose nanocomposite fibers according to an embodiment of the present technology.

[28] FIG. 2 is a flow chart comparing the plasma coagulation times of the halloysite coated cellulose nanocomposite fibers of FIG. 1 to QCG.

[29] FIG. 3A is a schematic chart of a wet-wet electrospinning process according to an embodiment of the present technology. FIG. 3B is an isometric view of the wet-wet electrospinning process of FIG. 3A.

[30] FIG. 4 is a chart showing a thermogravimetric analysis comparing the amounts of clay in various fiber composites.

[31] FIG. 5A is an SEM image of halloysite nanoclay at 50,000 magnification. FIG. 5B is an SEM image of halloysite nanoclay at 100,000 magnification. FIG. 5C is an EDX spectrum of halloysite nanoclay. FIG. 5D is an SEM image of kaolin clay at 50,000 magnification. FIG. 5E is an SEM image of kaolin clay at 100,000 magnification. FIG. 5F is an EDX spectrum of kaolin clay.

[32] FIG. 6A is an SEM image of an electrospun CNF at 100,000 magnification. FIG. 6B is an SEM image of an electrospun CHNF washed once at 100,000 magnification. FIG. 6C is an SEM image of a microfiber of QCG at 20,000 magnification. FIG. 6D is an SEM image of a microfiber of QCG washed once at 20,000 magnification. FIG. 6E is an EDX spectrum of an electrospun CNF. FIG. 6F is an EDX spectrum of an electrospun CHNF washed once. FIG. 6G is an EDX spectrum of a microfiber of QCG. FIG. 6H is an EDX spectrum of a microfiber of QCG washed once.

[33] FIG. 7A is a histogram chart of the diameter distribution of a CNF. FIG. 7B is a histogram chart of the diameter distribution of a CHNF. FIG. 7C is a histogram chart of the diameter distribution of a QCG.

[34] FIG. 8A is a surface area analysis chart showing nitrogen adsorption – desorption isotherms of CHNF. FIG. 8B is a surface area analysis chart showing nitrogen adsorption – desorption isotherms of QCG. FIG. 8C is a surface area analysis chart showing BET plots/Langmuir fits of CHNF. FIG. 8D is a surface area analysis chart showing BET plots/Langmuir fits of QCG.

[35] FIG. 9 is an aPTT assay chart showing the time taken to form a fibrin clot in a citrated plasma sample for CHNF, CHNF washed once, QCG, and QCG washed once.

[36] FIG. 10 is a plan view of a hemorrhage-treatment article according to an embodiment of the present technology.

DETAILED DESCRIPTION

[37] As used herein when describing a measuring numerical value, the term “about” includes the specific numerical value and a reasonable range encompassing the specific numerical value to account for, e.g., errors inherent in the measuring equipment used and in the humans handling such equipment. The reasonable range can be $\pm 5\%$, or even $\pm 10\%$ of the specific numerical value. Thus, for example, a recitation of “about 100 nm” could reasonably include the range of 90-110 nm without departing from the spirit and scope of the present technology.

[38] Referring now to **FIGs. 1-2**, some embodiments of the systems and methods of the present technology are directed to cellulose - nanoclay hemostatic nanocomposite fibers (“CHNF”). In some embodiments, the nanoclay is formed of an aluminosilicate clay. In preferred embodiments, the nanoclay is halloysite clay. In some embodiments, the fibers include nanocomposite fibers coated with halloysite, CHNFs **1**, such as that shown in **FIG. 1** after collection and freeze-drying. In some embodiments, the CHNFs **1** have an average diameter in the range of about 190 nm to about 710 nm. In some embodiments, the CHNFs **1** have an average diameter of about 450 nm. In some embodiments, the CHNFs **1** show 7-fold greater clay loading than QCG, and the CHNFs’ **1** small average diameter of 450 ± 260 nm

affords a greater specific surface area (about $33.6 \text{ m}^2\text{g}^{-1}$) than the larger average diameter of $12.6 \pm 0.9 \text{ }\mu\text{m}$ for QCG, which has a specific surface area of $1.6 \text{ m}^2\text{g}^{-1}$.

[39] Referring now to **FIGs. 3A-3B**, in some embodiments, the CHNFs **1** are fabricated using a one-step wet-wet electrospinning process, where **FIG. 3A** is an exemplary schematic of the process and **FIG. 3B** is an isometric view of an exemplary wet-wet electrospinning process. Halloysite ($\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4 \cdot 2\text{H}_2\text{O}$) is a naturally-occurring aluminosilicate nanoclay that exhibits a unique hollow tubular scroll structure. Halloysite is biocompatible and promotes blood coagulation. In some embodiments, cellulose is the matrix on which to immobilize the nanoclay due to its biocompatibility and low cost. Electrospinning can be used for preparing synthetic micro- and nano- polymeric fibers from solutions of polymers in volatile organic solvents. However, the biopolymer cellulose is a complex polysaccharide that does not dissolve in volatile organic solvents due to its extensive hydrogen-bonding network. Thus, in some embodiments, a non-volatile solvent is used for electrospinning cellulose. In preferred embodiments, a special class of non-volatile solvents, known as room temperature ionic liquids (“RTIL”), is used for electrospinning cellulose. The electrospinning of cellulose from a non-volatile RTIL, referred to as wet-wet electrospinning, is quite different from the conventional wet-dry electrospinning of a synthetic polymer from a volatile organic solvent. In wet-wet electrospinning, a biopolymer, such as cellulose, is dissolved in a non-volatile primary solvent, preferably an RTIL, which is miscible with a secondary solvent, such as water, alcohol (e.g., ethanol), or combinations thereof, in a coagulation bath collector. In some embodiments, this secondary solvent selected for use in the coagulation bath is a non-solvent of the polymer to promote its precipitation in the coagulation bath.

[40] In some embodiments, the wet-wet electrospinning technique was employed to produce cellulose nanofibers (“CNF”) and CHNFs **1** in a one-step process. In conventional wet-dry electrospinning, synthetic polymers are dissolved in volatile organic solvents, which rapidly evaporate upon the exit of the fiber jet from the needle forming a fiber mat on dry metal collectors. In contrast, in wet-wet electrospinning, a polymer is dissolved in a non-volatile primary solvent and electrospun into a coagulation bath collector containing a secondary non-solvent for the polymer. Primary solvent, in which the polymer is dissolved, is miscible with the secondary solvent so that the primary solvent is drawn away from the polymer resulting in its precipitation in the coagulation bath collector. Thus, in wet-wet

electrospinning fiber formation occurs through precipitation in the coagulation bath, rather than through the solvent evaporation occurring in wet-dry electrospinning.

[41] In some embodiments, cellulose was dissolved in RTIL and electrospun into a water bath. As cellulose coagulates it forms an intermediate hydrogel with water migrating into the fibers concomitant with RTIL diffusion into the water bath. The hydrogel ultimately collapses into CNFs that are collected and, in some embodiments, freeze-dried to obtain nonwoven cellulose nanofiber balls. In contrast, simple air-drying causes, in some embodiments, a collapse of morphology resulting in a cellulose mat. In some embodiments, the coagulation bath containing water is replaced with an aqueous suspension of halloysite nanoclay, resulting in the intermediate hydrogel entraining halloysite in the surface of the CNFs, and cellulose – halloysite nanofiber balls are collected by freeze-drying. This process physically embeds halloysite clay nanoparticles primarily on the surface of the fibers during the intermediate hydrogel state of cellulose in the coagulation bath. The freeze-dried CHNFs **1** appear identical to cotton balls but with an off-white color due to their halloysite surface, as shown in **FIG. 1**.

[42] In some embodiments, cotton balls (1.06 g) were mixed with 50.0 g of RTIL ([EMTM][Ac] density = 1.027 g/mL) using a magnetic stirrer at 80°C for 12 h to obtain a 2.12% (w/w) homogenous cellulose solution **2**. A halloysite nanoclay suspension **3** (10% (w/v)) was prepared by mixing 10 g of halloysite nanoclay in 100 mL of distilled water. The clay solution was then transferred to a large glass petri dish and placed on top of an orbital shaker **4**. A small piece of aluminum foil was placed inside the petri dish and connected to the negative lead of a high voltage supply using electrically insulated copper wires. In some embodiments, the high voltage electrospinning system is capable of generating a DC voltage up to 30 kV. The electrospinning parameters, i.e., needle diameter, distance between electrodes, polymer concentration, flow rate, etc., were optimized by an interactive approach.

[43] As shown in **FIG. 3A**, in some embodiments, the cellulose solution **2** was placed in a syringe (10 mL) that connected to a spinneret **5** with a fitted aluminum needle. The needle used has a blunt tip with an internal diameter of 0.635 mm (23 Gauge). Polytetrafluoroethylene tubing **6** delivered the cellulose solution **2** from the syringe to the spinneret **5**. The spinneret **5** was connected to the positive terminal of the high voltage supply. The distance between the tip of the aluminum needle and the surface of the clay solution **3** was fixed at 10 cm. Finally, a high voltage of 18 kV was applied between the

spinneret **5** set-up and the coagulation bath **7** as the cellulose solution **2** was pumped at a constant rate of 60 pL/min into the clay suspension **3** (in coagulation bath **7**) using, in some embodiments, a mechanical syringe pump. The orbital shaker **4** was run at 70 rpm to prevent sedimentation of clay on the bottom of the petri dish. In some embodiments, after the spinning process, the solidified cellulose- halloysite fibers were removed from the coagulation bath **7** and transferred into a clean 50% distilled water-ethanol bath to further remove ionic liquid from the fibers. In some embodiments, the fibers were freeze-dried to obtain the halloysite clay coated cellulose nanofibers, or CHNFs **1**, as shown in **FIG. 1**.

[44] In some embodiments, this electrospinning process was repeated using a distilled water coagulation bath instead of the halloysite nanoclay suspension to produce CNFs as a negative control. CNFs after electrospinning were mixed with halloysite clay ("MCNF") and used as a second control to evaluate the commonly used method of fiber production in the commercial products discussed above. This process for producing MCNFs involves immersing electrospun cellulose nanofibers in a halloysite nanoclay suspension (10% (w/v)) for 24 h. The MCNFs were then transferred into a clean 50% distilled water-ethanol bath for a washing step. Finally, the fibers were freeze-dried to obtain the halloysite clay mixed cellulose nanofibers, MCNFs. All of the electrospinning processes were carried out inside an anti-static polycarbonate box within a standard laboratory fume hood at $20\pm3^{\circ}\text{C}$ with a relative humidity controlled at $59\pm5\%$. In some embodiments, a digital humidity and temperature monitor was used to measure the temperature and the relative humidity.

[45] Referring now to **FIG. 4**, in some embodiments, thermogravimetric analysis shows that the CHNFs **1** left the higher residual mass at 1000°C when compared to MCNFs and QCG ($n=3$, $P<0.05$). The residual mass corresponds to halloysite nanoclay and the amount of clay present in the CHNFs was calculated to be $57.0 \pm 0.8\%$. In contrast, the MCNFs contained only $18.8 \pm 2.2\%$ halloysite nanoclay. Thus, the fabrication process according to the present technology is superior to the present commercial processes. Similarly, the amount of kaolin clay in QCG based on residual mass was $8.2 \pm 1.0\%$, and this amount was reduced to $1.6 \pm 0.5\%$ after a single washing step. Thus, in some embodiments, CHNFs **1** have approximately 7-times more active clay than QCG.

[46] The major mass loss of neat halloysite and kaolin clay occurred over the temperature range of $400\text{-}600^{\circ}\text{C}$, consistent with the dehydroxylation of structural Al-OH groups in the endothermic dehydration of clay. The CHNFs **1** showed the two characteristic

decompositions associated with combustion and smoldering of cellulose taking place at 200-350°C and 400-500°C, respectively. The purity of CHNFs **1** was substantiated by the absence of decomposition peaks associated hemicellulose (~220°C) and lignin (~100°C) impurities.

[47] Referring now to **FIGs. 5A-5F**, in some embodiments, morphology and the elemental composition of the clay samples were studied via SEM. **FIGs. 5A** and **5B** show SEM images of halloysite nanoclay at magnifications of 50,000 and 100,000, respectively. **FIGs. 5D** and **5E** show SEM images of kaolin clay at magnifications of 50,000 and 100,000, respectively. **FIGs. 5C** and **5F** show EDX spectra of halloysite nanoclay and kaolin clay, respectively. The scale bars are 1 μm (**FIGs. 5A** and **5D**) and 100 nm (**FIGs. 5B** and **5E**). In some embodiments, the halloysite nanoclay appeared to have the typical tubular morphology with diameters ranging from 30-120 nm. The SEM images showed the common plate morphology of kaolin clay, the clotting agent used in QCG (**FIGs. 5D** and **5E**). In contrast to halloysite nanoclay, kaolin clay flakes stack together to form large agglomerates (**FIG. 5D**). EDX analysis of both clay samples showed the same elemental composition as they are chemically identical (**FIGs. 5C** and **5F**).

[48] Referring now to **FIGs. 6A-7C**, in some embodiments, morphology and the elemental composition of the fiber samples were studied via SEM at image (**FIG. 6A**) and EDX spectrum (**FIG. 6E**) of an electrospun CHNF **1** at magnification of 100,000, SEM image (**FIG. 6B**) and EDX spectrum (**FIG. 6F**) of an electrospun CHNF **1** washed once at magnification of 100,000, SEM image (**FIG. 6C**) and EDX spectrum (**FIG. 6G**) of a microfiber of QCG at magnification of 20,000, and SEM image (**FIG. 6D**) & EDX spectrum (**FIG. 6H**) of a microfiber of QCG washed once at magnification of 20,000. The scale bars are 100 nm (**FIGs. 6A** and **6B**) and 1 μm (**FIGs. 6C** and **6D**). In some embodiments, electrospun cellulose nanofibers have a cylindrical structure (as shown in **FIG. 6A**) with an average diameter of 380 ± 140 nm (as shown in **FIG. 7A**). **FIGs. 7A-7C** show histograms of the diameter distribution of the fiber samples. Fiber diameter histograms of cellulose (**FIG. 7A**), CHNFs **1** (**FIG. 7B**) QCG (**FIG. 7C**) observed during SEM imaging ($P < 0.05$).

[49] The EDX spectrum of cellulose (**FIG. 6E**) reveals the characteristic K α x-ray peaks corresponding to carbon (0.277 keV) and oxygen (0.525 keV). CHNFs **1** were much smaller in size (**FIG. 6B**) with an average diameter of 450 ± 260 nm (**FIG. 7B**). In contrast,

QCG is composed of micron size fibers ranging from 11 to 15 μm with an average diameter of $12.6 \pm 0.9 \mu\text{m}$ (**FIG. 7C**).

[50] In preferred embodiments, the surface of CHNFs **1** is completely covered with halloysite nanoclay particles. In contrast, QCG has a sparse kaolin clay coating of the microfiber, as shown in **FIG. 6C**. As shown in **FIG. 6F**, the EDX spectrum of CHNFs **1** shows, in some embodiments, higher intensity K α x-ray peaks, for Al (1.487 keV) and Si (1.740 keV), than those observed in the EDX spectrum of QCG (**FIG. 6G**). The loss of significant amounts of kaolin clay after a single water wash was evident in the SEM image of washed QCG (**FIG. 6D**). This observation was confirmed by the absence of the characteristic Al and Si x-ray peaks in the EDX spectrum of washed QCG (**FIG. 6H**).

[51] In some embodiments, XRD analysis further supported the determinations made based on the TGA, SEM, and EDX studies discussed above. In some embodiments, the XRD pattern of CHNFs **1** was identical to the neat halloysite nanoclay, and peaks belonging to cellulose were not apparent. This verifies the abundance of halloysite nanoclay coating on the surface of the CHNFs **1**. In contrast, the XRD pattern of QCG showed broad peaks belonging to cellulose along with the two most intense peaks originating from the kaolin clay. These two peaks were not observed in the XRD pattern of the washed QCG. This supports the conclusion that most of the kaolin clay on the fiber was lost after a single washing step. Moreover, the crystallinity of both halloysite and kaolin clay was retained in the composite fibers.

[52] Referring now to **FIGs. 8A-8D**, in some embodiments, the specific surface area of the composite fibers was calculated using nitrogen adsorption - desorption isotherms (**FIGs. 8A-8B**) and the BET plots/Langmuir fits (**FIGs. 8C-8D**) of CHNFs **1** (**FIGs. 8A** and **8C**) and QCG (**FIGs. 8B** and **8D**). **FIG. 8A** shows adsorption plotting **8** and desorption plotting **9** of CHNFs **1**. **FIG. 8B** shows adsorption plotting **10** and desorption plotting **11** of QCG. CHNFs **1** showed significantly higher specific surface area ($33.6 \text{ m}^2\text{g}^{-1}$) than QCG ($1.6 \text{ m}^2\text{g}^{-1}$). This represents nearly 6-times the specific surface area of CNFs ($5.7 \text{ m}^2\text{g}^{-1}$). In some embodiments, the additional surface area of CHNFs **1** results from the nanoclay particles coating on the fiber surface. The specific surface area of the washed QCG was $3.5 \text{ m}^2\text{g}^{-1}$. This corresponds to approximately 125% increase in specific surface area of QCG. This likely results from the removal of large flaky agglomerates of kaolin clay from the QCG fibers during the washing step.

[53] The TGA, SEM, EDX, XRD, and BET characterization results all conclusively demonstrate the removal of kaolin clay from QCG even after a single washing step. Thus, in severe bleeding cases, or similar wetting in the field, the procoagulant clay particles are rapidly lost, reducing the procoagulant activity of QCG. In contrast, the CHNFs 1 show, in preferred embodiments, both a higher initial clay loading and remarkably less leaching of halloysite nanoclay particles from the fibers.

[54] In some embodiments, the procoagulant activity and stability of the CHNFs 1 were confirmed by plasma clotting studies using activated Partial Thromboplastin Time (“aPTT”) assay, which shows the time taken to form a fibrin clot in a citrated plasma sample. As shown in **FIG. 9**, the CHNFs 1 generate the fastest average plasma coagulation time (143 ± 19 seconds) of all samples tested. In some embodiments, the CHNFs 1 generate an average plasma coagulation time in the range of about 124 seconds to about 162 seconds. In some embodiments, the CHNFs 1 generate an average plasma coagulation time of about 143 seconds. This coagulation time is 2.4-times faster than that observed for QCG, which is 244 ± 41 seconds. Both CHNFs 1 and QCG performed better than CNFs, which took over 27 min to coagulate plasma. CHNFs 1 and QCG both showed reduced pro-coagulant performance after a single washing with water, although CHNFs 1 clotting performance reduced by only 24% compared to a 75% performance loss for QCG, as shown in **FIG. 9**. Additionally, the CHNF 1 composite in this embodiment had also been thoroughly washed with both water and ethanol in its preparation to remove residual ionic liquid. Thus, it is not surprising that additional washing step of the CHNFs 1 with water resulted in only a slight reduction in procoagulant activity. An additional advantage of CHNFs 1 is that the average plasma coagulation time of halloysite clay is 55 seconds faster than kaolin clay, thus making halloysite nanoclay superior to kaolin.

Examples

[55] In some embodiments, the CHNFs 1 formed by the wet-wet electrospinning process discussed above are thoroughly washed with alcohol-water mixture to remove residual RTIL. QCG, marketed by Z-MEDICA, EEC, approved by the United States Food and Drug Administration and also endorsed by the US Department of Defense for external use due to its ability of controlling severe arterial hemorrhage, was selected as the commercial standard to compare the procoagulant efficacy of these CHNFs 1.

[56] In some embodiments, pure absorbent cotton balls, HPLC grade room temperature ionic liquid (RTIL), 1-ethyl-3-methylimidazolium acetate ([EMIM][Ac], >95.0%), absolute ethanol (>99.8%), and halloysite nanoclay were used. In some embodiments, kaolin clay and QCG were used. In some embodiments, citrated human plasma and an aPTT clinical diagnostic kit were used. In some embodiments, double-distilled water from an in-house water purification system was used.

[57] In some embodiments, CHNFs 1, MCNFs, CNFs, and QCG were subjected to thermogravimetric analysis to deduce the amount of clay present in the fiber composites. The composite fibers before and after a water wash were also analyzed along with halloysite and kaolin clay controls. All the samples were heated from room temperature (25°C) to 1000°C at a constant heating rate of 10°C/min under dry atmospheric conditions.

[58] In some embodiments, a field emission scanning electron microscope with energy dispersive X-ray spectroscopy was used to investigate the morphology and the elemental composition of the CHNFs 1. QCG was imaged before and after subjecting to a washing step to empirically evaluate the leaching of kaolin clay from the fibers. CNFs, halloysite nanoclay, and kaolin clay were also subjected to SEM and EDX analysis as controls. The average fiber diameters were calculated from 100 individual fibers from 10 identical electrospinning experiments were employed in this fiber diameter analysis.

[59] In some embodiments, crystallinity of the clay in the CHNFs 1 and QCG were studied using an X-ray diffractometer, and were compared to the halloysite and kaolin clay controls. The crystallinity of the CNFs was also evaluated.

[60] In some embodiments, the surface area of the CHNFs 1 was estimated by conducting a BET surface area analysis. A sample of CHNFs (117 mg) was degassed at 100°C for 24 h. This was followed by N₂ adsorption-desorption at 77.35 K (-196°C) under a relative vapor pressure (P/P₀) of 0.05-0.3. An electrospun CNF sample (70 mg) was used as a control in this experiment. QCG samples before (447 mg) and after (241 mg) undergoing a washing step was also subjected to surface area analysis using same experimental conditions as CHNFs.

[61] In some embodiments, an aPTT assay was employed to evaluate human plasma coagulation performance of the CHNFs 1, QCG, clay controls, and CNFs using citrated human plasma. This assay is a two-step process that measures the time taken to form

a fibrin clot in a citrated plasma sample. A sample of citrated human plasma (1 mL) was incubated with ~1 mg of fiber or clay sample (the activator) for 5 min followed by the addition of 500 μ L of 25 mM calcium chloride solution at 37°C. A stopwatch was turned on immediately upon the introduction of the calcium chloride solution. An inoculating loop was used to detect the formation of the fibrin clot and the time taken to form the fibrin clot was recorded. This process was repeated with CHNFs 1 and QCG before and after subjecting to a washing step to evaluate the depletion of coagulation performance due to the loss of clay from the composite fibers. A positive control experiment was conducted using 200 μ L of citrated human plasma, 100 μ L of calcium chloride, and 100 μ L of the particle-based Kontakt activator (contains 1.2% rabbit brain phospholipid, 0.03% magnesium aluminum silica, 0.4% phenol, 0.8% buffer, salt, and stabilizers) of the aPTT assay. In some embodiments, the above clotting experiments were run in borosilicate glass culture tubes.

[62] The composite fibers according to embodiments of the present technology will make a dramatic, lifesaving impact in the treatment of hemorrhages, such as those associated with traumatic injuries, surgical procedures, and frequent bleeding events. In some embodiments, the CHNFs 1 outperformed the present leading commercial product, QCG, as a topical blood clotting material by exhibiting 2.4-times faster plasma coagulation time. In some embodiments, the CHNFs 1 had three-times the clay anti-leaching performance compared with the QCG post-wetting. The drastic reduction of human plasma coagulation time renders the CHNFs 1 a potential life-saving material. The advantages of using the nanocomposites of the present technology over the commercially available products are that the materials used for fabrication are cheap, naturally occurring, and the CHNFs 1 retains its function for a longer period of time. In some embodiments, the CHNFs 1 have superior anti-leaching of clay with three-times higher post-wetting clotting activity compared to QCG. Halloysite clay is also more effective in plasma coagulation than commercial kaolin clay. The physical and thermal properties of the CHNFs were evaluated using scanning electron microscopy, energy dispersive X-ray spectroscopy, X-ray diffraction, Brunauer-Emmett-Teller surface area analysis, and thermogravimetric analysis. In some embodiments, the CHNFs 1 show 7-fold greater clay loading than QCG and their small average diameter of 450 ± 260 nm compared to 12.6 ± 0.9 μ m for QCG accounts for part of this increase in surface area. This affords a 22-times greater specific surface area ($33.6 \text{ m}^2 \text{ g}^{-1}$) than QCG ($1.6 \text{ m}^2 \text{ g}^{-1}$).

[63] In some embodiments, the wet-wet electrospinning process relying on a coagulation bath containing the clay suspension, as discussed above, obtained high clay loadings and increased specific surface areas. Fibers electrospun into the clay suspension had 3-fold higher clay loading than when CNFs were simply immersed in halloysite clay suspension to prepare MCNFs. The choice of halloysite nanoclay in place of kaolin clay further enhanced performance. Neat halloysite nanoclay coagulates human plasma approximately 1.6-times faster than neat kaolin clay. Thus, a combination of higher surface area supplied by the electrospun fibers, and enhanced clotting performance of the halloysite nanoclay, increased the overall plasma clotting performance of the CHNFs **1**.

[64] In some embodiments, the CHNFs **1** retained over three-times the clotting activity compared with QCG after washing once with water, losing only 24% of its clotting activity compared to a 75% loss of procoagulant activity observed for QCG. This stability of CHNFs **1** is extremely important for stopping traumatic external hemorrhages on the battlefield and in the operating room since continuous blood flow greatly reduces the performance of QCG. When QCG was subjected to washing in distilled water, nearly 80% of its clay content was quickly lost. In some embodiments, the intermediate hydrogel state formed in the coagulation bath during the wet-wet electrospinning process facilitates kinetic entrapment of halloysite nanoclay particles, resulting in higher clay retention of the CHNFs **1**. This physical embedding of nanoclay particles during the electrospinning process does not occur with simple immersion of prefabricated CNFs in a clay suspension, which is the process used to produce QCG.

[65] In some embodiments, a CHNF **1** made according to the wet-wet electrospinning process discussed above is used as an anti-hemorrhagic agent. In some embodiments, CHNF **1** is placed on or within a hemorrhage-treatment article **12**, as shown in **FIG. 10**. The hemorrhage-treatment article **12** is used to apply the CHNF **1** to a hemorrhage to coagulate the plasma in animal blood, preferably mammal blood, and more preferably human blood. In some embodiments, the hemorrhage-treatment article **12** is designed for external, topical use, such as a wipe, pad, gauze, bandage, wrap, etc.

[66] Although the technology has been described and illustrated with respect to exemplary embodiments thereof, it should be understood by those skilled in the art that the foregoing and various other changes, omissions, and additions may be made there and thereto, without departing from the spirit and scope of the present technology.

CLAIMS

What is claimed is:

1. A method of making a cellulose – nanoclay hemostatic nanocomposite fiber, comprising the steps of:
 - preparing a cellulose solution including cellulose and a non-volatile solvent;
 - preparing a nanoclay suspension including an aluminosilicate and water;
 - electrospinning the cellulose solution into a first bath including the nanoclay suspension; and
 - removing solidified cellulose – nanoclay fibers from the first bath.
2. The method of claim 1, wherein the non-volatile solvent is a room temperature ionic liquid.
3. The method of claim 1, wherein the cellulose solution is homogenous.
4. The method of claim 1, wherein the aluminosilicate is halloysite.
5. The method of claim 1, wherein the nanoclay suspension further includes alcohol.
6. The method of claim 1, further comprising the step of:
 - transferring the solidified cellulose – nanoclay fibers to a second bath including alcohol and water.
7. The method of claim 1, further comprising the step of:
 - freeze-drying the solidified cellulose – nanoclay fibers.
8. The method of claim 6, further comprising the steps of:
 - removing the solidified cellulose – nanoclay fibers from the second bath; and
 - freeze-drying the solidified cellulose – nanoclay fibers.
9. The method of claim 1, wherein the solidified cellulose – nanoclay fibers have an average diameter of about 190 nm to about 710 nm.
10. The method of claim 1, wherein the solidified cellulose – halloysite fibers have a specific surface area of about $33.6 \text{ m}^2\text{g}^{-1}$.
11. The method of claim 1, wherein the solidified cellulose – nanoclay fibers are configured to coagulate human plasma in an average time of about 124 seconds to about 162 seconds.

12. A cellulose – nanoclay hemostatic nanocomposite fiber made by the method of claim 1, wherein the nanoclay is halloysite.
13. An anti-hemorrhagic agent, comprising:
 - a matrix of cellulose nanofibers; and
 - a plurality of halloysite nanoclay particles attached to the matrix of cellulose nanofibers;wherein the anti-hemorrhagic agent is configured to coagulate human plasma in an average time of about 143 seconds.
14. The anti-hemorrhagic agent of claim 13, wherein the plurality of halloysite nanoclay particles are attached to the matrix of cellulose nanofibers via a wet-wet electrospinning process.
15. The anti-hemorrhagic agent of claim 14, wherein the wet-wet electrospinning process comprises the steps of:
 - preparing a homogenous cellulose solution including cellulose and a room temperature ionic liquid;
 - preparing a nanoclay suspension including halloysite and distilled water;
 - electrospinning the cellulose solution into a first bath including the nanoclay suspension;
 - transferring solidified cellulose – halloysite fibers from the first bath to a second bath including ethanol and distilled water;
 - removing the solidified cellulose – halloysite fibers from the second bath; and
 - freeze-drying the solidified cellulose – halloysite fibers.
16. The anti-hemorrhagic agent of claim 13, further comprising an article for applying the anti-hemorrhagic agent to a hemorrhage of a human user.
17. A system of coagulating human plasma, comprising:
 - an article comprising a cellulose – halloysite hemostatic nanocomposite fiber; and
 - a human user having a hemorrhage;wherein the article is configured to apply the cellulose – halloysite hemostatic nanocomposite fiber to the hemorrhage.
18. The system of claim 17, wherein the cellulose – halloysite hemostatic nanocomposite fiber is made by a wet-wet electrospinning process.

19. The system of claim 18, wherein the wet-wet electrospinning process comprises the steps of:
 - preparing a homogenous cellulose solution including cellulose and a room temperature ionic liquid;
 - preparing a nanoclay suspension including halloysite and distilled water;
 - electrospinning the cellulose solution into a first bath including the nanoclay suspension;
 - transferring solidified cellulose – halloysite fibers from the first bath to a second bath including ethanol and distilled water;
 - removing the solidified cellulose – halloysite fibers from the second bath; and
 - freeze-drying the solidified cellulose – halloysite fibers.
20. The system of claim 17, wherein the cellulose – halloysite hemostatic nanocomposite fiber is configured to coagulate human plasma in an average time of about 143 seconds.

1/13

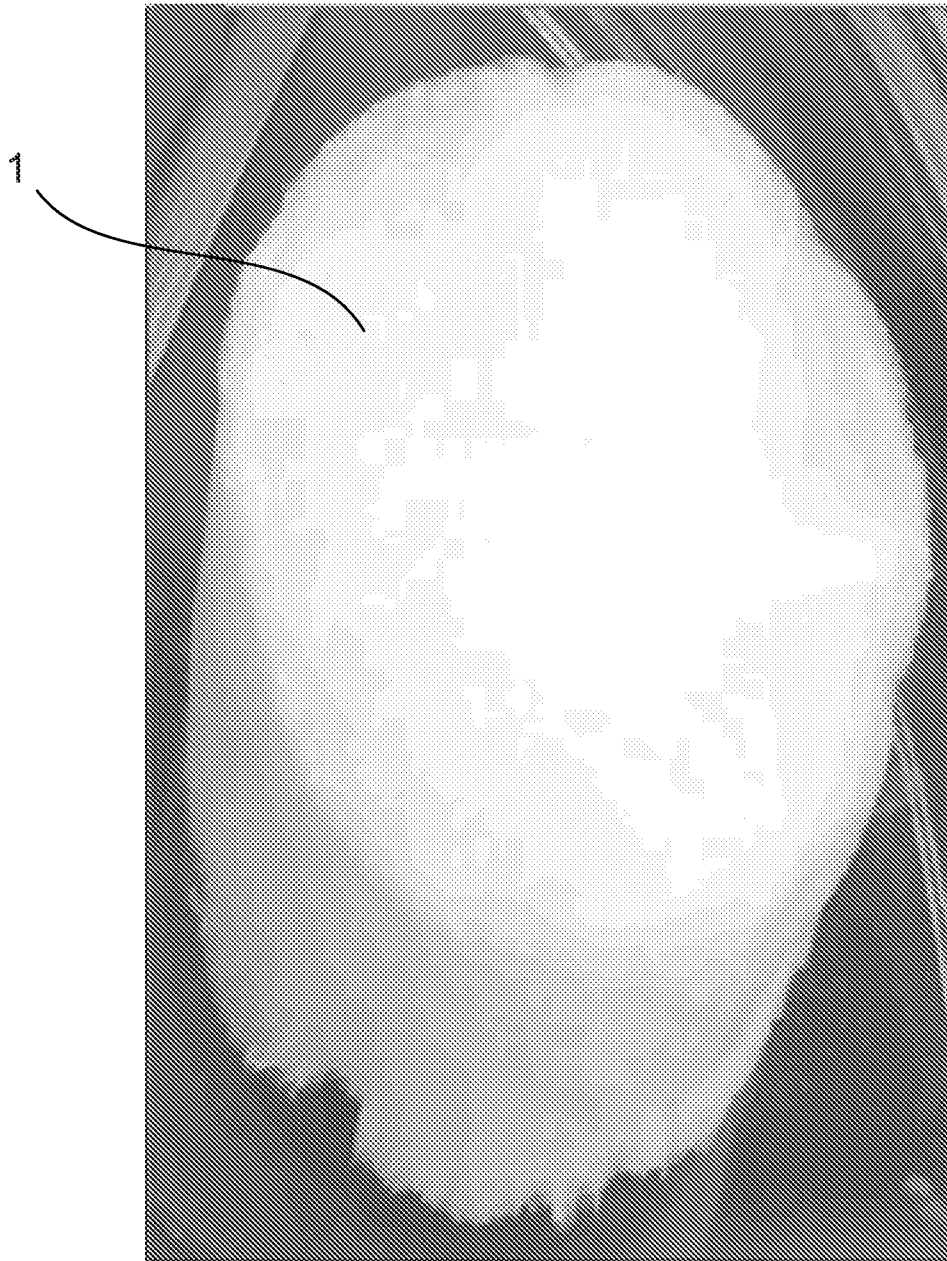


FIG. 1

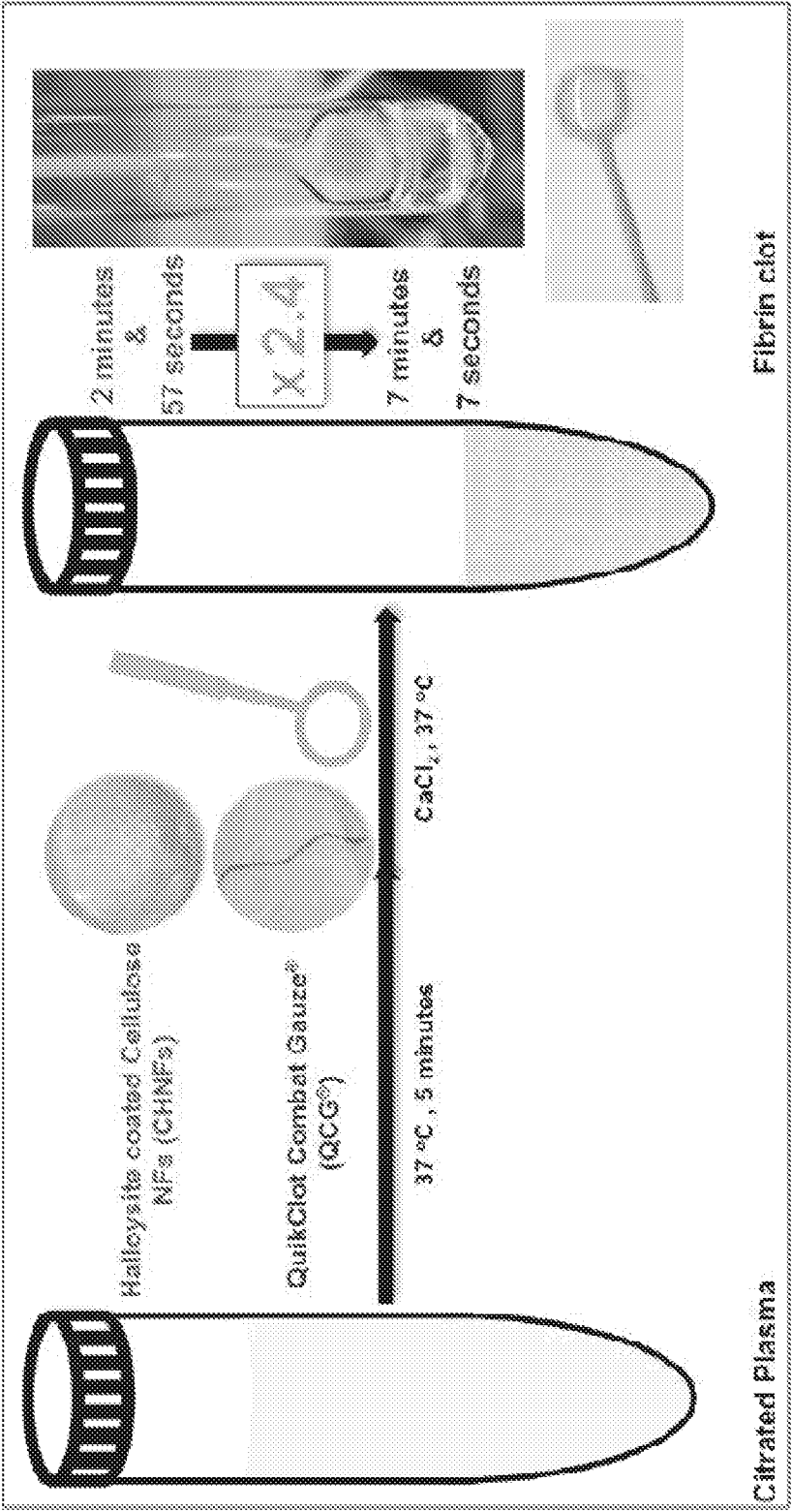


FIG. 2

3/13

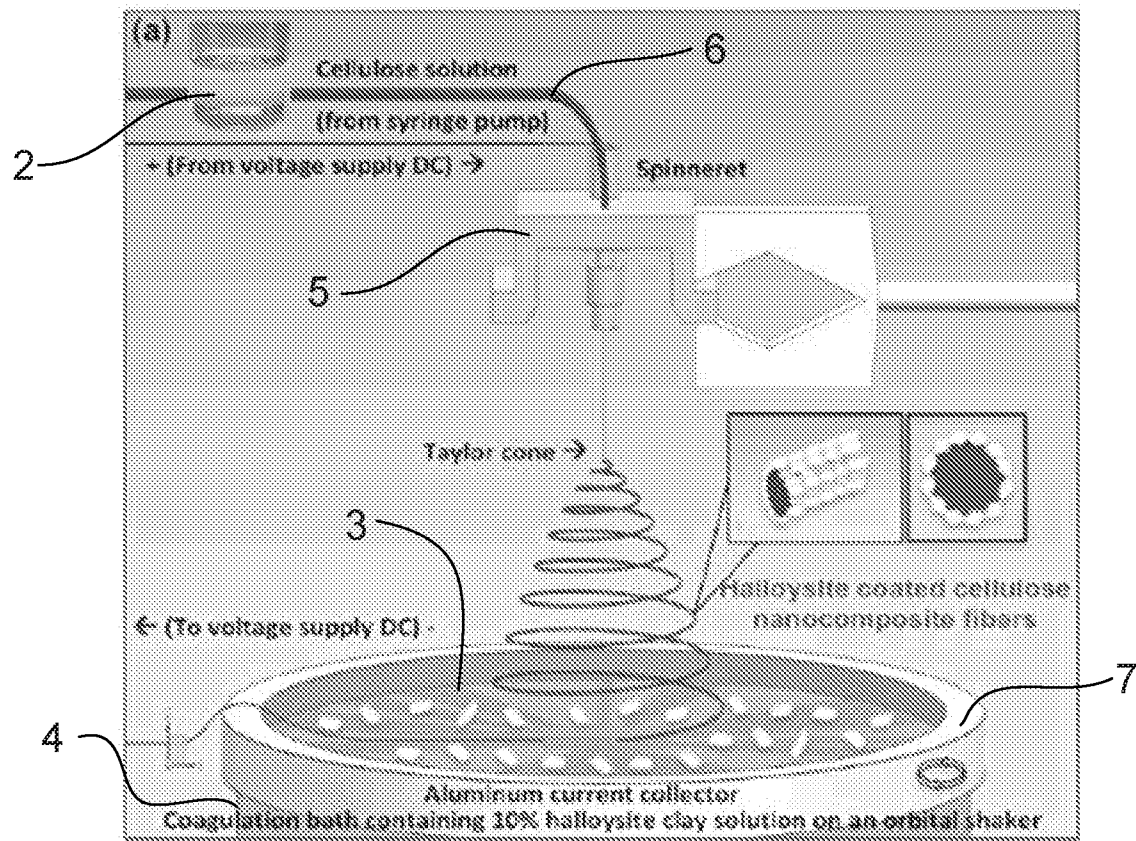


FIG. 3A

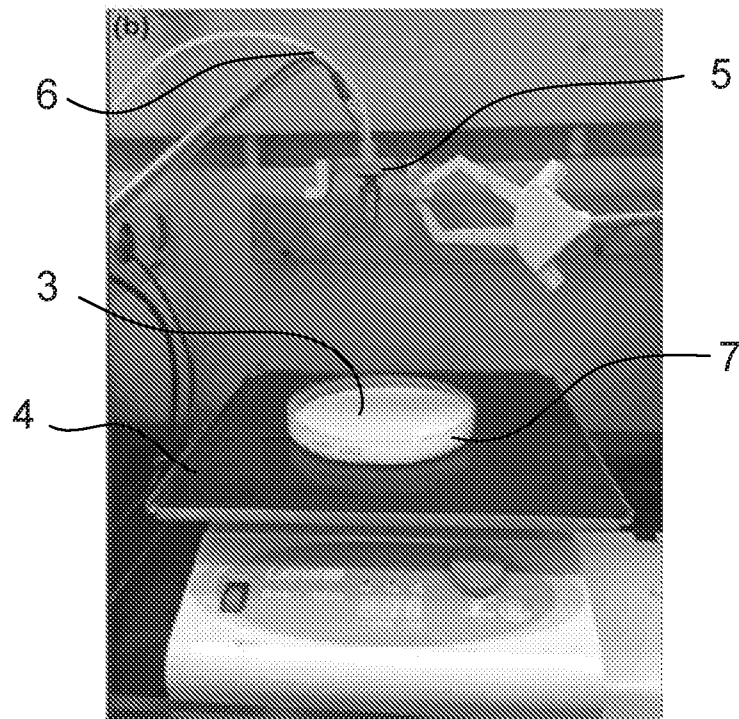


FIG. 3B

4/13

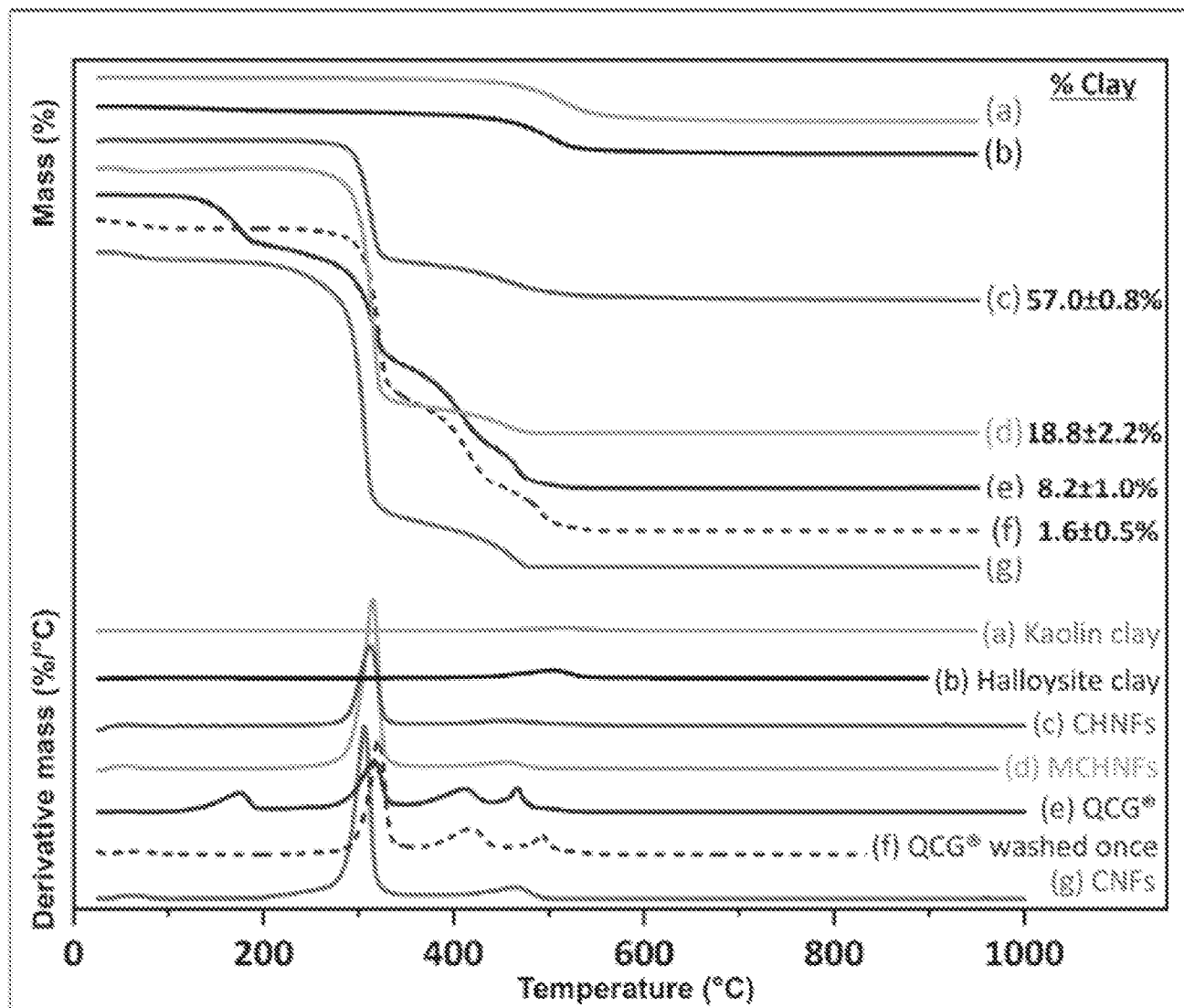


FIG. 4

5/13

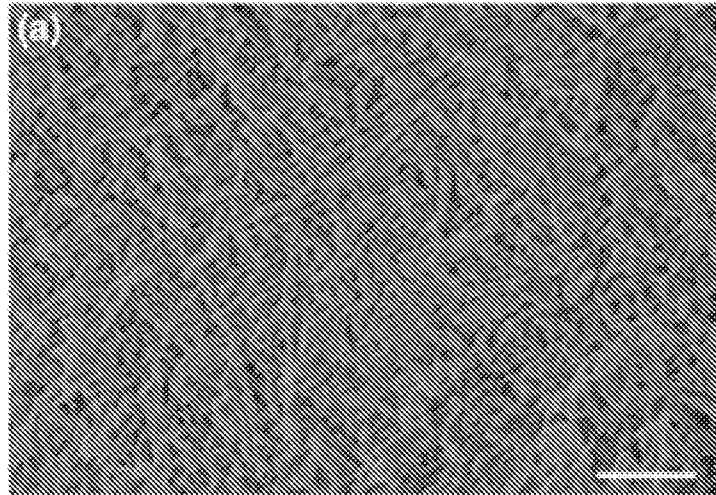


FIG. 5A

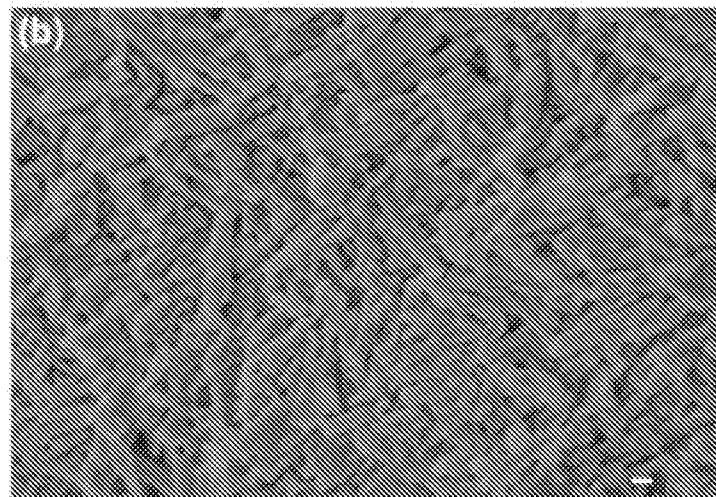


FIG. 5B

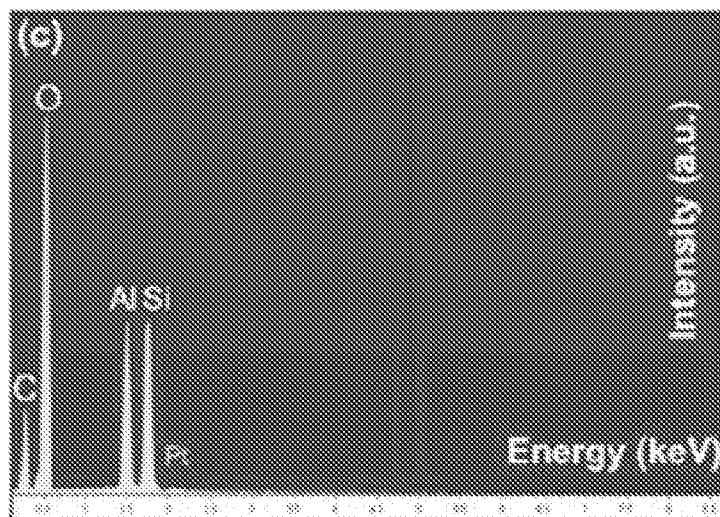


FIG. 5C

6/13

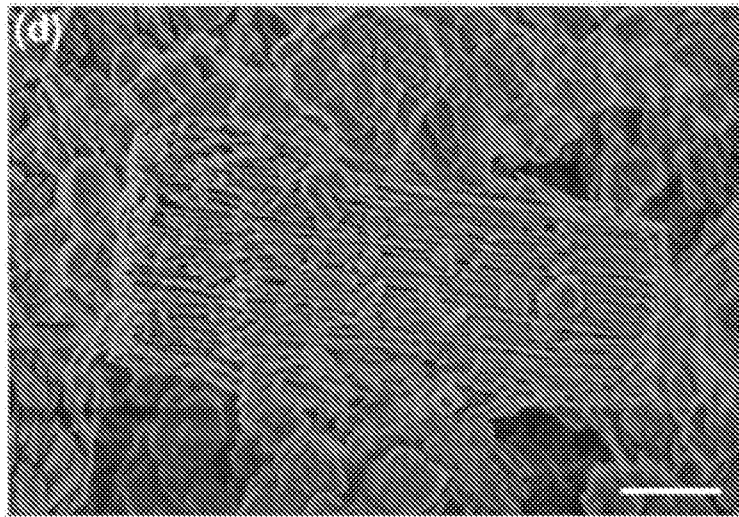


FIG. 5D

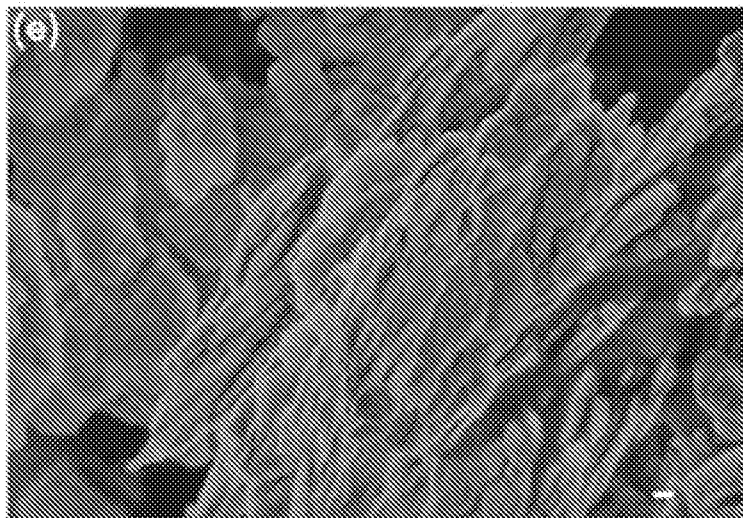


FIG. 5E

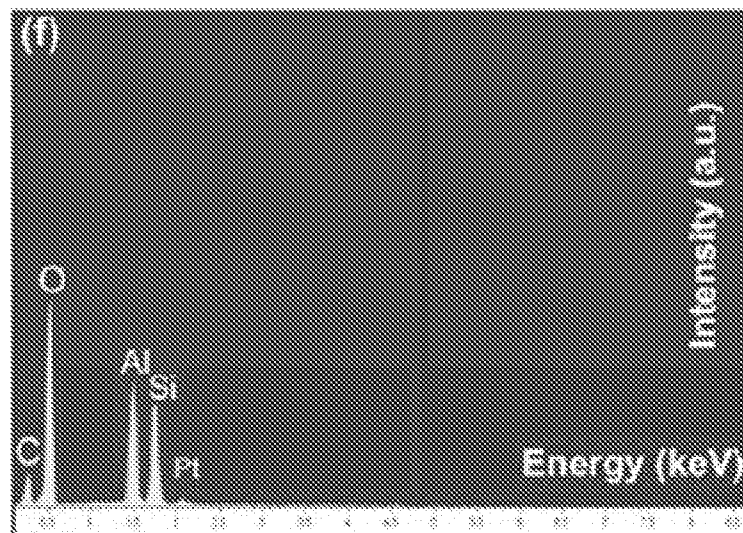


FIG. 5F

7/13

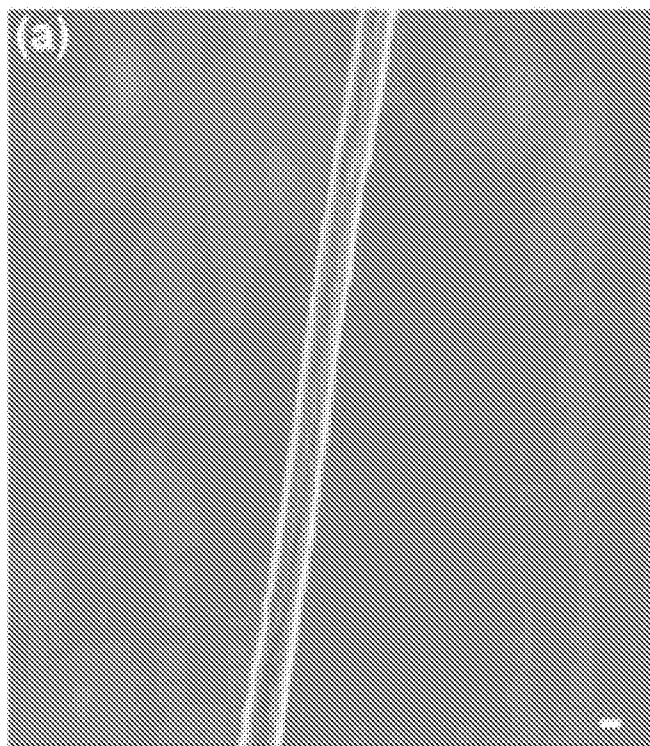


FIG. 6A

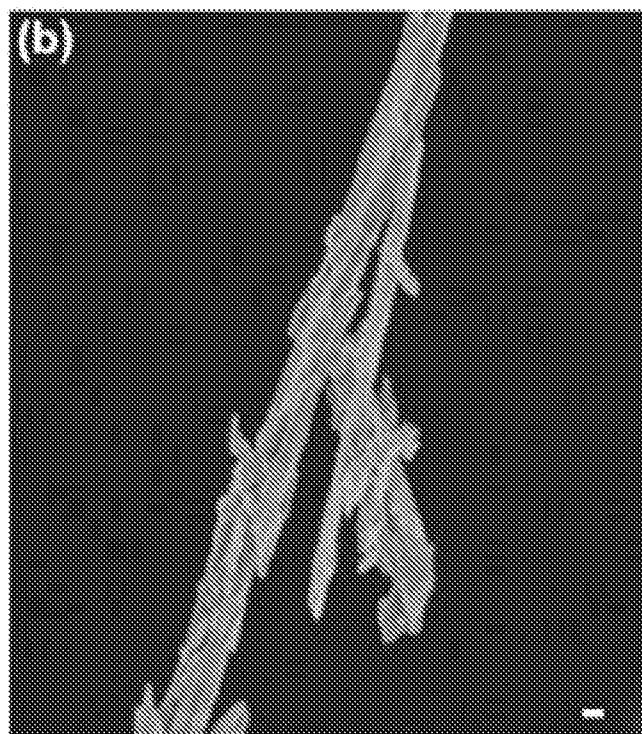


FIG. 6B

8/13

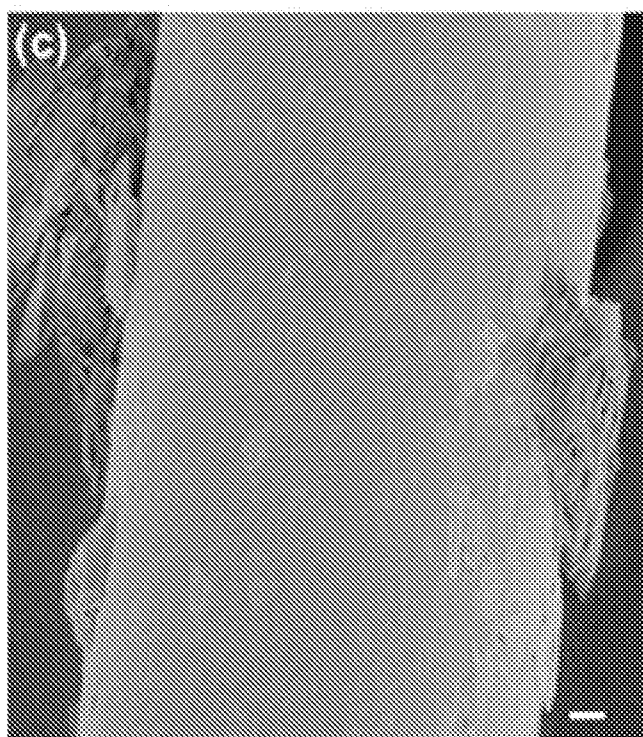


FIG. 6C

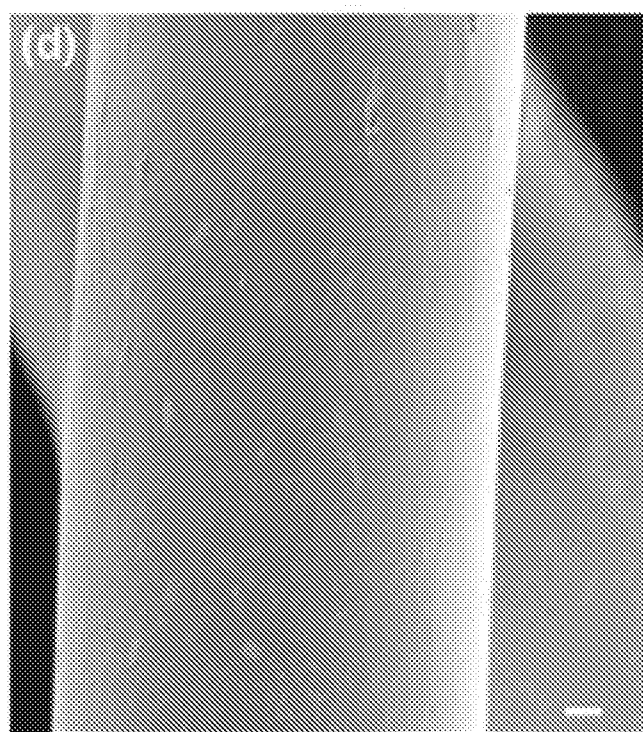


FIG. 6D

9/13

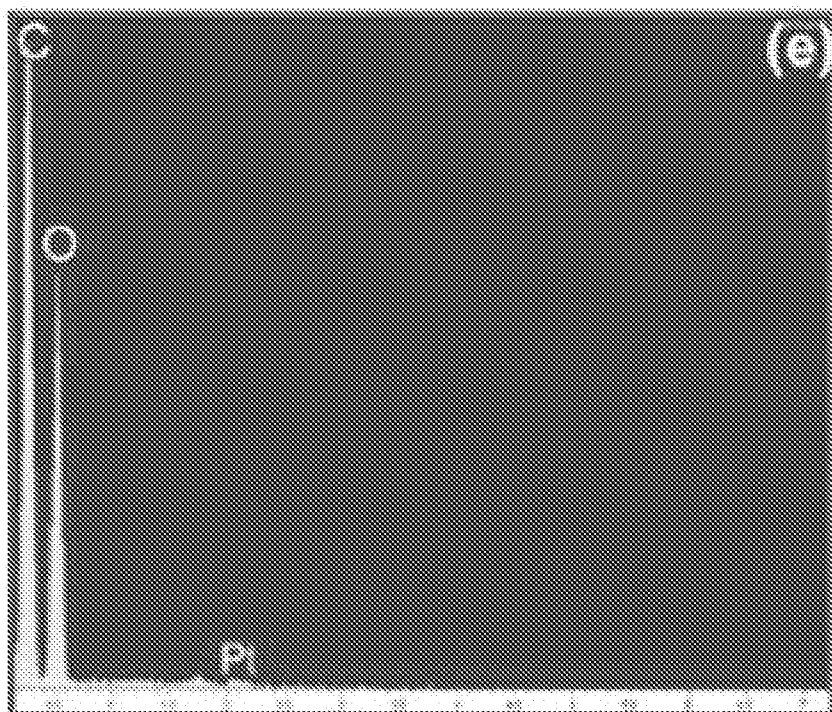


FIG. 6E

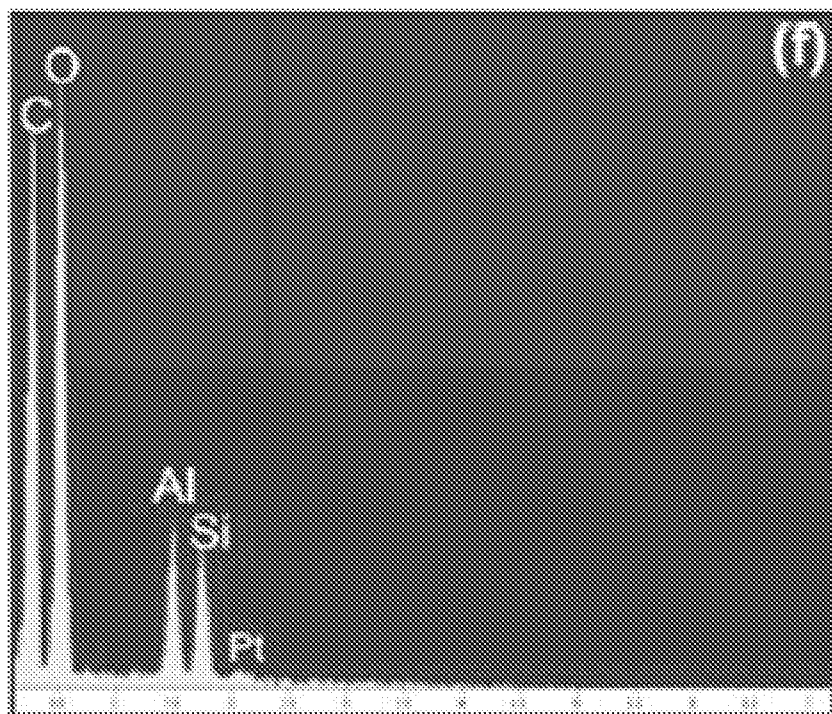


FIG. 6F

10/13

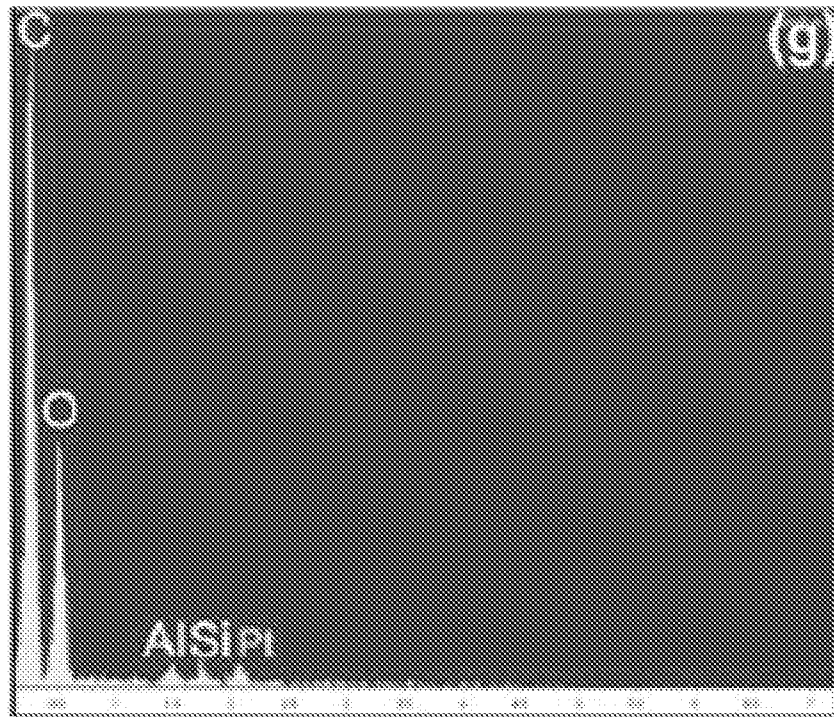


FIG. 6G

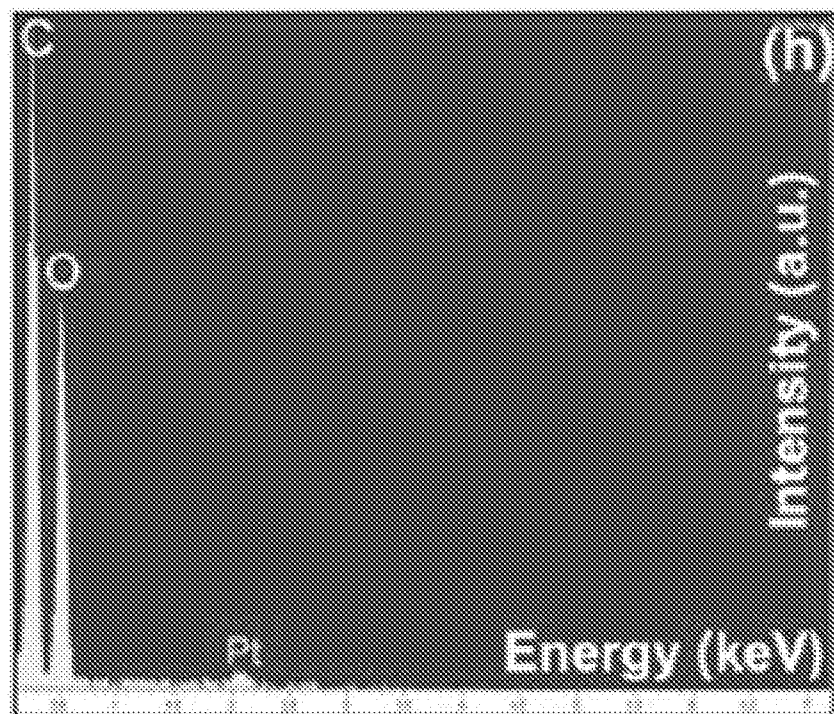


FIG. 6H

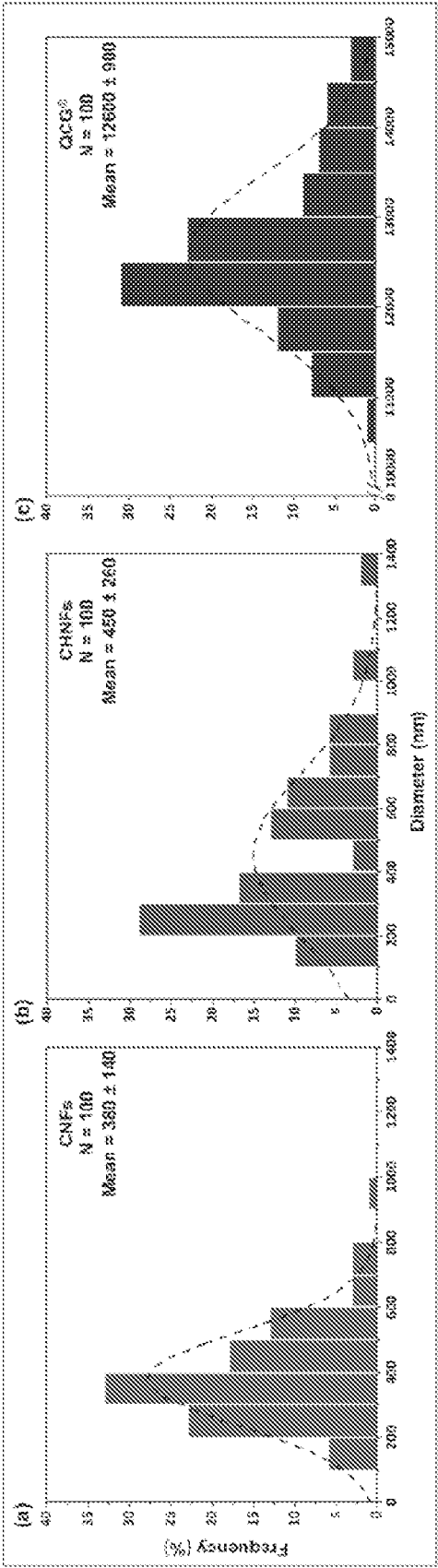


FIG. 7A

FIG. 7B

FIG. 7C

12/13

FIG. 8A

FIG. 8B

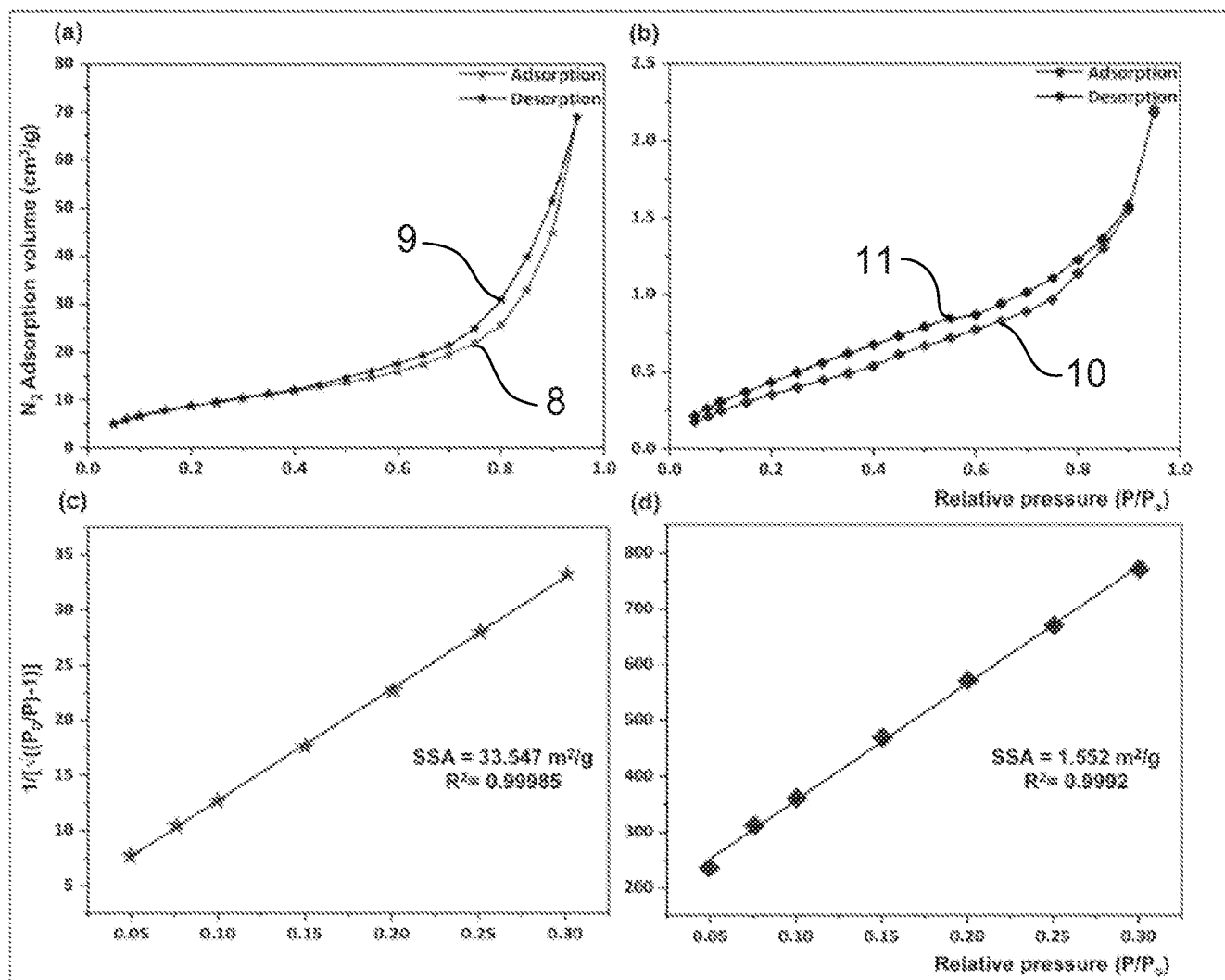


FIG. 8C

FIG. 8D

13/13

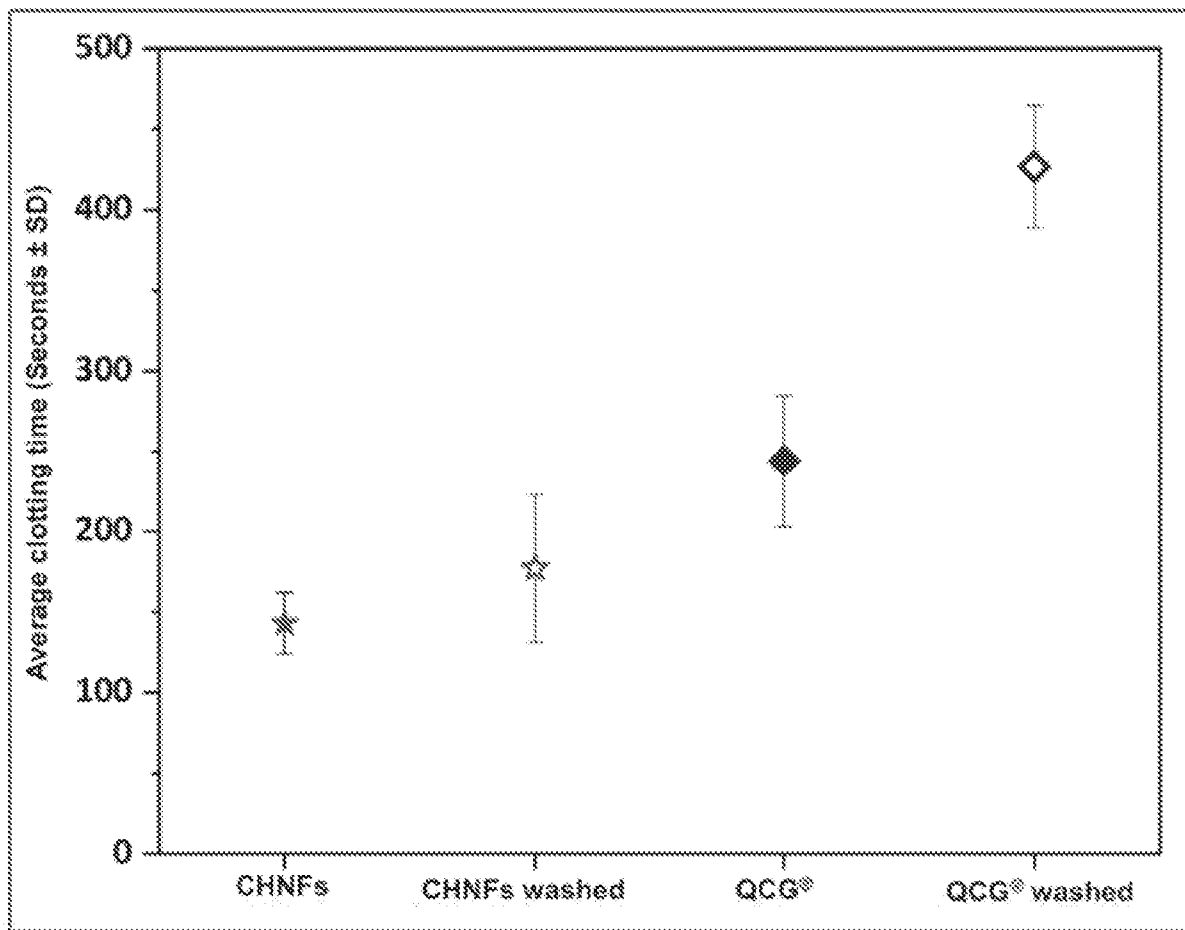


FIG. 9

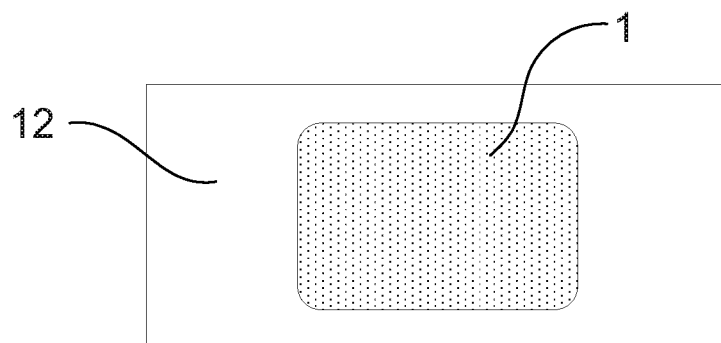


FIG. 10

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US20/16119

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61L 15/12, 15/16 (2020.01)

CPC - A61K 9/5161, 9/5192; A61L 15/08, 15/12, 15/125, 15/16, 15/18, 15/22, 15/225, 15/28; B82Y 30/00; D01D 5/0007, 5/0053, 5/0046; D04H 1/7288; D01F 2/02, 8/02

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)

See Search History document

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

See Search History document

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

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2015/097047 A1 (SAPPI NETHERLANDS SERVICES B.V.) 02 July 2015; page 3 lines 28-29, page 4 lines 10-15, page 5 lines 15-16, page 7 lines 29-31, and page 8 lines 6-11	1-20
A	US 2014/0027938 A1 (BOARD OF TRUSTEES OF THE UNIVERSITY OF ALABAMA) 30 January 2014; paragraphs [0049], [0129]	1-20
A	US 2011/0165811 A1 (DOW CORNING CORPORATION) 07 July 2011; paragraphs [0015], [0037], [0044]	1-20
A	US 2012/0259262 A1 (Z-MEDICA CORPORATION) 11 October 2012; paragraph [0044]	1-20
A	US 2015/0306274 A1 (MEDTRADE PRODUCTS LIMITED) 29 October 2015; paragraphs [0012], [0014], [0044]	1-20

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

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"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

19 March 2020 (19.03.2020)

Date of mailing of the international search report

23 APR 2020

Name and mailing address of the ISA/US

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