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(54) Title: FUNCTIONALIZED ENVIRONMENTALLY BENIGN NANOPARTICLES

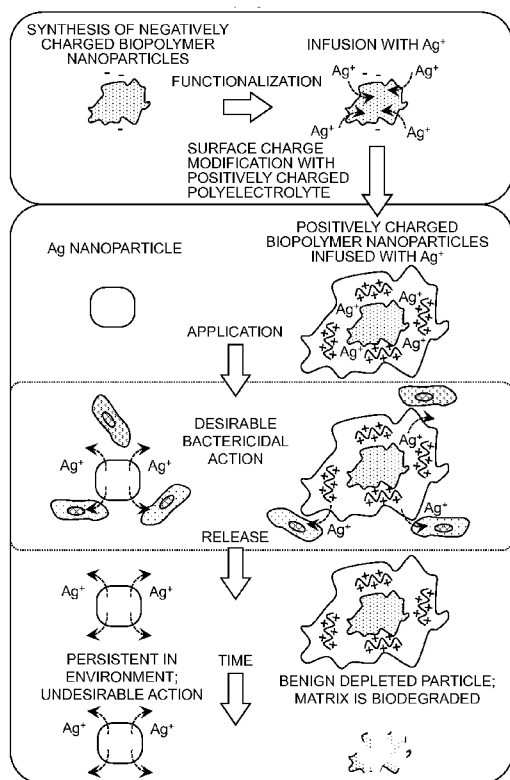


Figure 1

(57) Abstract: Provided herein are internally and/or externally functionalized environmentally benign nanoparticles (EbNPs), as well as the preparation and applications thereof, which may be produced by a three step procedure: (1) synthesis of native EbNPs, (2) functionalization with active agents, and (3) additional surface property customization via one or more modifier(s).



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FUNCTIONALIZED ENVIRONMENTALLY BENIGN NANOPARTICLES

STATEMENT OF FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with government support under Grant No. 554,871 awarded by the U.S. Environmental Protection Agency. The government has certain rights in the invention.

FIELD OF THE INVENTION

This invention relates generally to the preparation and applications of internally and/or externally functionalized environmentally benign nanoparticles (EbNPs), which are produced by a three-step procedure: (1) synthesis of native EbNPs, (2) functionalization with active agents, and (3) additional surface property customization via one or more modifier(s).

BACKGROUND OF THE INVENTION

Engineered nanoparticles exhibit unique and useful physical, chemical, and biological particle-specific attributes that may help to solve pressing challenges of mankind in industries including life sciences, energy, and health care. However, nanoparticle waste has been recognized as a potential health hazard (Bystrzejewska-Piotrowska et al., Nanoparticles: Their potential toxicity, waste and environmental management. *Waste Management* **2009**, 29 (9), 2587-2595), as the post-utilization activity of engineered nanoparticles combined with their persistence may result in short and long-term toxicity in humans and the environment (Stern et al., Nanotechnology Safety Concerns Revisited. *Toxicological Sciences* **2008**, 101 (1), 4-21). For example, it has been found that the physical and chemical characteristics of persistent nanoparticles (PNPs), and therefore, their activity, may not change even after high-temperature treatments in solid-waste incineration plants (Walser et al., Persistence of engineered nanoparticles in a municipal solid-waste incineration plant. *Nat Nano* **2012**, advance online publication).

Silver nanoparticles (AgNPs) are among the most widely employed PNPs, as their broad-spectrum antimicrobial properties allow them to combat bacteria strains exhibiting antibiotic resistance (Panáček et al., Silver Colloid Nanoparticles: Synthesis, Characterization, and Their Antibacterial Activity. *The Journal of Physical Chemistry B* **2006**, 110 (33), 16248-16253), which are reported in human pathogens including *Escherichia coli* (*E.coli*) (Cohen, S. N.; Chang, A. C. Y.; Hsu, L., Nonchromosomal Antibiotic Resistance in Bacteria: Genetic Transformation

of *Escherichia coli* by R-Factor DNA. *Proceedings of the National Academy of Sciences* **1972**, 69 (8), 2110-2114) and *Pseudomonas aeruginosa* (*P. aeruginosa*) (Poole et al., Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. *Journal of Bacteriology* **1993**, 175 (22), 7363-7372). As infection control measures can minimize the spread of drug-resistant bacteria (Carmeli et al., Health and economic outcomes of antibiotic resistance in *pseudomonas aeruginosa*. *Archives of Internal Medicine* **1999**, 159 (10), 1127-1132), and, therefore, the potential for nosocomial infections (Cohen, M. L., Epidemiology of Drug Resistance: Implications for a Post-Antimicrobial Era. *Science* **1992**, 257 (5073), 1050-1055), silver-containing products may find increasing utilization in the medical sector to prevent bacteria growth on catheters (Samuel et al., Prevention of catheter-related infections: the potential of a new nano-silver impregnated catheter. *Int J Antimicrob Ag* **2004**, 23, Supplement 1 (0), 75-78), prostheses (Gosheger et al., Silver-coated megaendoprostheses in a rabbit model—an analysis of the infection rate and toxicological side effects. *Biomaterials* **2004**, 25 (24), 5547-5556), and dental materials (Ohashi et al., Antibacterial activity of silver inorganic agent YDA filler. *Journal of Oral Rehabilitation* **2004**, 31 (4), 364-367), and to reduce the infection potential of burn wounds (Klasen, H. J., A historical review of the use of silver in the treatment of burns. II. Renewed interest for silver. *Burns* **2000**, 26 (2), 131-138).

In addition, with the emergence of antimicrobial PNPs in textiles (Lee et al., Antibacterial effect of nanosized silver colloidal solution on textile fabrics. *J Mater Sci* **2003**, 38 (10), 2199-2204), water filters (Jain et al., Potential of silver nanoparticle-coated polyurethane foam as an antibacterial water filter. *Biotechnol Bioeng* **2005**, 90 (1), 59-63), and other consumer products, the human exposure potential to PNPs with their associated risks increases. Human skin exposure studies indicate that AgNPs can be released from antibacterial fabrics into liquids resembling "sweat" (Kulthong et al., Determination of silver nanoparticle release from antibacterial fabrics into artificial sweat. *Particle and Fibre Toxicology* **2010**, 7 (1), 8). Studies on commercially available wound dressings proved that dressings containing AgNPs exhibit stronger cytotoxic effects toward keratinocytes than do PNP-free counterparts (Paddle-Ledinek et al., Effect of Different Wound Dressings on Cell Viability and Proliferation. *Plastic & Reconstructive Surgery. Current Concepts in Wound Healing*. **2006**, 117 (7S)). *In vitro* studies on mammalian fibroblasts have revealed that AgNP can induce apoptosis. In this context, AgNP may potentially affect human health (Arora et al., Cellular responses induced by silver nanoparticles: *In vitro* studies. *Toxicology Letters* **2008**, 179 (2), 93-100; Arora et al., Interactions of silver nanoparticles with primary mouse fibroblasts and liver cells. *Toxicology*

and Applied Pharmacology 2009, 236 (3), 310-318; Ahamed et al., Silver nanoparticle applications and human health. Clinica Chimica Acta 2010, 411 (23-24), 1841-1848).

Several methods for the preparation of antimicrobial silver-based nanoparticle systems have been reported. Most procedures employ highly reactive reducing agents such as sodium borohydride (NaBH_4) or hydrazine (N_2H_4) to reduce silver ions to metallic silver. Green synthesis methods of producing AgNP can reduce the environmental impact during fabrication given that no harsh solvents or reducing agents are employed (Kumar et al., Silver-nanoparticle-embedded antimicrobial paints based on vegetable oil. *Nat Mater* **2008**, 7 (3), 236-241; Raveendran et al., Completely "Green" Synthesis and Stabilization of Metal Nanoparticles. *J Am Chem Soc* **2003**, 125 (46), 13940-13941). However, due to the persistent nature of AgNPs, the problem of post-utilization toxicity associated with non-degradable nanoparticles remains unaddressed.

SUMMARY OF THE INVENTION

The present invention provides a functionalized environmentally benign nanoparticle (EbNP). The nanoparticle may include: a biodegradable biopolymer core, a bioactive agent such as an antiviral or cytotoxic agent loaded on the biodegradable core, and a bioadhesive layer coating the biodegradable core loaded with the bioactive agent.

In some embodiments, the antiviral or cytotoxic agent is loaded or otherwise associated with the biodegradable core by infusion, absorption and/or physical and/or chemical adsorption. In some embodiments, the antiviral or cytotoxic agent is loaded or otherwise associated with the biodegradable core by adsorption. In some embodiments, the antiviral or cytotoxic agent is loaded or otherwise associated with the biodegradable core by electrostatic attraction.

In some embodiments, the bioadhesive layer is coated or otherwise associated with the biodegradable core loaded with the antiviral or cytotoxic agent by infusion, absorption and/or physical and/or chemical adsorption. In some embodiments, the bioadhesive layer is coated or otherwise associated with the biodegradable core loaded with the antiviral or cytotoxic agent by adsorption. In some embodiments, the bioadhesive layer is coated or otherwise associated with the biodegradable core loaded with the antiviral or cytotoxic agent by electrostatic attraction.

In some embodiments, the biodegradable biopolymer core is a lignin or a modified lignin. In some embodiments, the biodegradable biopolymer core is a byproduct of lignin degradation (e.g., humic acid). In some embodiments, the lignin is not crosslinked.

In some embodiments, the biodegradable biopolymer core is an anionic polymer. In some embodiments, the biodegradable biopolymer core is a plant- or animal-derived biopolymer, e.g.,

a cellulose, a chitin, a chitosan (e.g., a medium or high molecular weight chitosan or a derivative thereof), a hemicellulose, a lignocellulose, a modified cellulose (e.g., cellulose acetate, cellulose nitrate, cellulose propionate, ethyl cellulose, hydroxyethyl cellulose, hydroxypropyl methylcellulose phthalate, hydroxypropyl methylcellulose acetate succinate, or methyl cellulose, or a derivative thereof), a modified chitosan (e.g., a low molecular weight chitosan, a chitosan with amino groups in the backbone, or a derivative thereof), a modified lignin (e.g., sulfonated (Indulin C) or unsulfonated (Indulin AT, High Purity Lignin) lignin), a protein (e.g., a prolamin (such as gliadin, hordein, secalin, zein, kafirin or avenin) or a gluten (such as gliadin or glutenin), or a combination thereof.

In some embodiments, the biodegradable biopolymer core is a synthetic polyphenol, a natural phenolic polymer, or a linear, branched, or cross-linked polysaccharide, or a derivative thereof.

In some embodiments, the biodegradable biopolymer core is a linear, branched, or cross-linked biopolymer of natural or technical origin (i.e., physically or chemically modified during processing of natural compounds and appearing usually as bi-product or waste stream of such process).

In some embodiments, the biodegradable biopolymer core is a large molecular weight polyphenol or natural phenolic polymer, such as lignin or modified lignin or a combination thereof.

In some embodiments, the antiviral or cytotoxic agent is a biocide, a cationic metal, a catalyst, a fumigant (e.g., 1,3-dichloropropene, chloropicrin, formaldehyde, iodoform, metam sodium, methyl bromide, methyl iodide, methyl isocyanate, phosphine, or sulfuryl fluoride), an herbicide (e.g., glyphosate, triclopyr, 1,1'-dimethyl-4,4'-bipyridinium ion (paraquat) or a chemical derivative, analogue or salt thereof; or a chloracetanilide herbicide (such as acetochlor, alachlor, butachlor, metolachlor, or propachlor), glyphosate herbicide, an imidazolinone herbicide (such as imazapyr, imazamethabenz-methyl, imazapic, imazethapyr, imazamox or imazaquin), an organic herbicide (such as corn gluten meal, vinegar, D-limonene, or monocerin), a phenoxy herbicide (such as 2,4-Dichlorophenoxyacetic acid, 2,4,5-Trichlorophenoxyacetic acid, 2-Methyl-4-chlorophenoxyacetic acid, 2-(2-Methyl-4-chlorophenoxy)propionic acids, 2-(2,4-Dichlorophenoxy)propionic acid, or 2,4-Dichlorophenoxy)butyric acid), a phenylurea herbicide (such as *N*'-(3,4-dichlorophenyl)-*N,N*-dimethylurea (diuron), 1,1-dimethyl-3-[3-(trifluoromethyl)phenyl] (fluometuron), or *N,N*-dimethyl-*N*'-[4-(1-methylethyl)phenyl] (isoproturon)), a triazine herbicide (such as ametryn, atrazine, cyanazine, prometon, prometryn, propazine, simazine, terbuthylazine, or terbutryn), or a triazolopyrimidine herbicide (such as

clorasulam-methyl, diclosulam, florasulam, flumetsulam, metosulam, penoxsulama, or pyroxsulama)), a pesticide (e.g., an avicide, an insecticide (such as a carbamate insecticide (such as aldicarb, carbaryl, carbofuran, formentanate, methiocarb, oxamyl, pirimicarb, propoxur, or thiodicarb) or a pyrethroid insecticide (such as allethrin, bifenthrin, cyhalothrin, lambda-cyhalothrin, cypermethrin, cyfluthrin, deltamethrin, etofenprox, fenvalerate, permethrin, phenothrin, prallethrin, psmethrin, tetramethrin, tralomethrin, or transfluthrin)), a miticide, a molluscicide, a nematocide, or a rodenticide (such as an anticoagulants, brodifacouma, bromadiolonea, chlorophacinone, difethialone, diphacinone, pindone, warfarin, nonanticoagulant, bromethalin, cholecalciferol, strychnine, or zinc phosphide)), a photocatalyst, or a semiconductor (e.g., Ag_2S , CdS , CdSe , CdTe , Cu_2S , CuCl , CuO , Fe_2O_3 , Fe_2S , Fe_3O_4 (α , β , γ , ϵ), FeO , NiO , TiO_2 , ZnO , ZnS , ZnSe , or ZnTe). In some embodiments, the biocide is an algaecide, a bactericide (e.g., an anionic antimicrobial peptide or an anionic antimicrobial protein (AAMP); or an antibacterial peptide (such as gramicidin, magainins, melittin, defensins, amphotericins or nystatin)), bactericidal/permeability-increasing protein (BPI), or a fungicide (e.g., captan, chlorothalonil, cyrodinil, folpet, mepanipyrim, pyrimethanil, sulfur, or vinclozolin; or an ethylenebisdithiocarbamate (such as mancozeb, maneb, metiram, nabam, or zineb) or a natural fungicide (such as *ampelomuces quisqualis*, cinnamaldehyde, cinnamon essential oil, jojoba oil, monocerin, neem oil, rosemary oil, or tee tree oil); or bifonazole, butoconazole clomidazole, clotrimazole, croconazole, econazole, fenticonazole, ketoconazole, isoconazole, miconazole, neticonazole, omoconazole, oxiconazole, sertaconazole, sulconazole, tioconazole, fosfluconazole, terconazole, fluconazole hexaconazole, isavuconazole, itraconazole, posaconazole, voriconazole, albaconazole, abafungin, hamycin, natamycin, nystatin, amphotericin, hamycin, amorolfine, butenafine, naftifine, terbinafine, amorolfine, anidulafungin, caspofungin, micafungin, Anidulafungin, flucytosine, or griseofulvin). In some embodiments, the biocide is 6-phthalimidohexaneperoxoic acid, 2-pyridinecarboxylic acid, or 1-Hexadecylpyridinium chloride; or a phenol, 2-phenylphenol, pidedyldimethylammonium chloride, chloroxylenol, hexachlorophene, thymol, amylmetacresol, methylbenzethonium chloride, cetalkonium chloride, cetylpyridinium chloride, cetrimonium, cetrimide, dofanium chloride, tetraethylammonium bromide, didecyldimethylammonium chloride, domiphen bromide, or zinc pyrithione.

In some embodiments, the antiviral or cytotoxic agent is a cationic metal (e.g., Ag^+ , Ag^{2+} , Ag^{3+} , Co^{2+} , Cu^{2+} , Fe^{2+} , Ni^{2+} , or Zn^{2+}). In some embodiments, the metal is in substantially ionic form (e.g., at least 20, 30, 40, 50, 60, 70, 80, or 90 % or more by weight of the metal is present in ionic form versus metallic form).

In some embodiments, the bioadhesive layer comprises a cationic polymer. In some embodiments, the cationic polymer is a polyamino-polymer. In some embodiments, the polyamino-polymer is branched polyethyleneimine (BPEI), polyallylamine hydrochloride (PAH), polydiallyldimethylammonium chloride (PDADMAC), polyethoxylated tallow amine (POEA), polyethyleneimine (PEI), or polylysine. In some embodiments, the bioadhesive layer comprises primary, secondary, tertiary, or quaternized amines (benzalkonium chloride, benzethonium chloride). In some embodiments, the bioadhesive layer comprises carbohydrates, polypeptides, lectins, proteins, or antibodies or other molecules or materials with affinity to microbes, viruses or seeds. In some embodiments, the bioadhesive layer comprises nanohairs or nanolatches. In some embodiments, the bioadhesive layer comprises a silicone based surfactant (e.g., silicone blends with polysiloxane chains). In some embodiments, the bioadhesive layer is an oil based surfactant (e.g., a vegetable oil, methylated vegetable oil, seed oil, crop oil, petroleum based oil, silicon based oil, or a blend of these). In some embodiments, the bioadhesive layer is an amphiphilic protein, soy protein, fluorescence DNA, or a fluorescence marker. In some embodiments, the bioadhesive layer is poly(hexamethylenebiguanide) hydrochloride (PHMB), polyaminopropyl biguanide (PAPB), 1-octanamine, 1-hexadecylpyridinium chloride, or poly-(D)glucosamine. In some embodiments, the bioadhesive layer is a chitosan.

In some embodiments, the nanoparticle has a diameter of about 10 nm to about 500 nm, or about 20 nm to about 100 nm, or about 50 nm to about 80 nm.

Also provided herein is a coated article comprising a surface wherein at least a portion of the surface is coated with a functionalized environmentally benign nanoparticle as taught herein.

In some embodiments, the coated article is an air filter, an article of clothing, an article of hygiene, a building material, a face mask, a food stuff package, a medical device, or a seed. In some embodiments, the medical device is bandage, a biological implant, a dressing, a medical scaffold, a surgical instrument, or a wound covering.

Also provided is the use of a functionalized environmentally benign nanoparticle as taught herein to impart antiviral or cytotoxic properties to a substrate.

Also provided is a method of controlling (e.g., inhibiting growth of, killing or otherwise reducing) one or more microorganisms or other environmental pests or hazards, comprising the step of applying a nanoparticle as taught herein to a substrate or environment (e.g., land, crops, bodies of water, etc.) in a treatment effective amount.

Further provided are methods for fabricating a functionalized environmentally benign nanoparticle as taught herein. In some embodiments, the method may include: contacting a

solvent containing a dissolved biodegradable biopolymer with an anti-solvent so as to form a biodegradable biopolymer core; loading an antiviral or cytotoxic agent on the biopolymer core; and coating the biopolymer core and the antiviral or cytotoxic agent with a bioadhesive layer. In some embodiments, the method may include: altering the pH of a suitable solvent containing a dissolved biodegradable biopolymer so as to form a biodegradable biopolymer core; loading an antiviral or cytotoxic agent on the biopolymer core; and coating the biopolymer core and the antiviral or cytotoxic agent with a bioadhesive layer. In some embodiments, the method may include: contacting an organic solvent containing a dissolved biodegradable biopolymer with an aqueous solvent under suitable pH conditions so as to form a biodegradable biopolymer core; loading an antiviral or cytotoxic agent on the biopolymer core; and coating the biopolymer core and the antiviral or cytotoxic agent with a bioadhesive layer. In some embodiments, the method may include: contacting an aqueous containing a dissolved biodegradable biopolymer with a polyelectrolyte under suitable conditions so as to form a biodegradable biopolymer core; loading an antiviral or cytotoxic agent on the biopolymer core; and coating the biopolymer core and the antiviral or cytotoxic agent with a bioadhesive layer. In some embodiments, the solvent may comprise acetone, ethylene glycol, propylene glycol, glycerol, methyl acetate, ethyl acetate, methanol, ethanol, hexanol, petrol ether, toluene, benzene, turpentine, dichloromethane, chloroform, formic acid, isopropanol, polyethylene oxide, or a combination thereof.

Still further provided is a functionalized environmentally benign nanoparticle fabricated according to any of the methods as taught herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Schematic of the concept for making and using environmentally benign bactericidal nanoparticles (EbNPs) compared to the present use of AgNPs according to embodiments of the present invention.

Figure 2: Schematics of the concept for synthesizing HPL EbNP via the solvent – antisolvent method according to embodiments of the present invention.

Figure 3: HPL EbNP synthesis according to embodiments of the present invention. **a)** size as a function of initial HPL concentration in solvent. **b)** TEM micrographs of native HPL EbNP.

Figure 4: HPL EbNP size and ζ -potential as a function of final pH according to embodiments of the present invention.

Figure 5: Schematics of the IAT EbNP synthesis methods (Frangville, C.; Rutkevicius, M.; Richter, A. P.; Veleev, O. D.; Stoyanov, S.; Paunov, V. N., Fabrication of Environmentally

Biodegradable Lignin Nanoparticles. ChemPhysChem 2012, 13) according to embodiments of the present invention.

Figure 6: Results of IAT EbNP synthesis via pH drop as a function of acid added according to embodiments of the present invention.

5 **Figure 7:** IAT EbNPs size and ζ -potential vs. amount of HNO_3 added to 5 ml IAT0.5 wt% in ethylene glycol according to embodiments of the present invention.

Figure 8: IAT EbNP size and ζ -potential vs. pH according to embodiments of the present invention.

10 **Figure 9:** IAT EbNP dissolution as a function of pH, obtained by UV-Vis measurements, according to embodiments of the present invention.

Figure 10: IAT EbNP stability as a function of ionic strength according to embodiments of the present invention.

15 **Figure 11:** DLVO modeling of IAT EbNP system according to embodiments of the present invention. The interaction energy in kT is modeled as a function of particle separation distance in nm for three different ionic strengths.

Figure 12: Infusion of native IAT EbNPs according to embodiments of the present invention. a, TEM micrograph of native IAT EbNP. b, Schematics showing native IAT EbNP infusion with Ag^+ in aqueous solution. c, TEM image of silver-ion infused IAT EbNP.

20 **Figure 13:** Langmuir adsorption isotherm fit according to embodiments of the present invention. Ag^+ adsorption on IAT EbNP nanoparticles normalized on m^2 particle surface area vs. initial Ag^+ loading per m^2 particle surface area.

Figure 14: EbNP diameter and ζ -potential vs. initial PDADMAC wt% according to embodiments of the present invention.

25 **Figure 15:** EbNP diameter and ζ -potential vs. initial PAH wt% according to embodiments of the present invention.

Figure 16: CFU on reference plate without agent (left) and CFU on test plate with antimicrobial agent (right) according to embodiments of the present invention.

30 **Figure 17:** Wet method schematic for antimicrobial testing according to embodiments of the present invention. (1) placement of active agent into centrifugal tubes, (2) addition of PBS buffer, (3) addition of bacteria solution, vortexing for 1 minute and 30 minutes, plating after 1 minute and 30 minutes, incubation of samples, and investigation of CFUs.

Figure 18: Qualitative *E. coli* test – CFU reduction efficiency of selected IAT EbNP, BPEI AgNP, and AgNO_3 samples according to embodiments of the present invention. a, after 1 minute incubation time. b, after 30 minutes incubation time.

Figure 19: Quantitative *Pseudomonas aeruginosa* test – CFU reduction efficiency of selected BPEI AgNP, AgNO₃, PDADMAC, and IAT EbNP samples according to embodiments of the present invention. **a**, after 1 minute incubation time. **b**, after 30 minutes incubation time.

Figure 20: Schematics of the concept for making and using environmentally benign bactericidal nanoparticles (EbNPs) compared to the present use of AgNPs according to embodiments of the present invention.

Figure 21: Qualitative *E. coli* test – CFU reduction efficiency of selected IAT EbNP, BPEI AgNP, and AgNO₃ samples according to embodiments of the present invention. **a**, after 1 minute contact time. **b**, after 30 minutes contact time.

Figure 22: Quantitative *Pseudomonas aeruginosa* test – CFU reduction efficiency of selected IAT EbNP, BPEI AgNP, and AgNO₃ samples according to embodiments of the present invention. **a**, after 1 minute contact time. **b**, after 30 minutes contact time.

Figure 23: Schematic of the hypothesis of the antimicrobial mechanism of f-EbNPs-PDADMAC according to embodiments of the present invention. **a**, Ag-EbNP-PDADMAC are electrostatically attracted to bacteria cell and **b**, can deliver the silver ions leading to cell death.

DETAILED DESCRIPTION OF EMBODIMENTS

In order to minimize the post-utilization hazard of nanoparticles, provided herein are environmentally-degradable nanoparticles designed to minimize their residence time and presence in the environment. In some embodiments, the degradable nanoparticles have matching functionality to persistent nanoparticles (PNPs) and may serve as a suitable solution to the problem of post-utilization toxicity with PNPs.

Lignin, the most abundant aromatic polymer in nature (Lora, J. H.; Glasser, W. G., Recent Industrial Applications of Lignin: A Sustainable Alternative to Nonrenewable Materials. *Journal of Polymers and the Environment* **2002**, *10* (1), 39-48), has an amorphous structure and is biodegradable in the environment by micro-organisms such as fungi and bacteria. Lignin covalently crosslinks the cell walls of plants, and plays a vital role in plant health, growth, and development (Iiyama, K.; Lam, T.; Stone, B. A., Covalent Cross-Links in the Cell Wall. *Plant Physiol.* **1994**, *104* (2), 315–320).

When extracted from biomass, the structure of modified lignin varies depending on the initial plant source and the method of isolation. Lignin obtained via the organosolv process, such as High Purity Lignin (HPL), is strongly hydrophobic, does not incorporate any sulfur containing groups, and therefore, preserves best the structure of native lignin of all processed lignins (Glasser et al., The chemistry of several novel bioconversion lignins. *J Agr Food Chem* **1983**, *31* (5), 921-930).

However, the most common extraction method is the Kraft pulping processes (Chakar, F. S.; Ragauskas, A. J., Review of current and future softwood kraft lignin process chemistry. *Industrial Crops and Products* **2004**, 20 (2), 131-141). Indulin AT (IAT), a modified lignin that contains a small number of hydrophilic thiol groups, is recovered by this process.

5 In aqueous systems, matrixes of IAT have shown high absorbance capabilities of heavy metal ions for environmental remediation purposes (Guo et al., Adsorption of metal ions on lignin. *Journal of Hazardous Materials* **2008**, 151 (1), 134-142; Harmita et al., Copper and cadmium sorption onto kraft and organosolv lignins. *Bioresource Technology* **2009**, 100 (24), 6183-6191). Cationic metal ions are electrostatically attracted to IAT, which is negatively charged in aqueous
10 solution due to deprotonation of its main functional groups. pH-stable IAT-based environmentally benign nanoparticles (EbNPs) may be synthesized in ethylene glycol (Frangville, C.; Rutkevicius, M.; Richter, A. P.; Velez, O. D.; Stoyanov, S.; Paunov, V. N., Fabrication of Environmentally Biodegradable Lignin Nanoparticles. *ChemPhysChem* **2012**, 13).

As taught herein, IAT EbNPs may be infused with functional metal ions to synthesize
15 degradable nanoparticles to provide the nanoparticle functionality of the respective metal PNPs, while increasing post-utilization safety.

In some embodiments, the metal ions are provided in a substantially ionic form, rather than metallic form. For example, in some embodiments at least 20, 30, 40, 50, 60, 70, 80, or 90 % or more by weight of the metal is present in ionic form versus metallic form.

20 The schematic in Figure 1 illustrates three steps involved in generating exemplary sustainable antimicrobial nanoparticles, and their life cycle in comparison to that of persistent AgNPs. In one, non-limiting embodiment, the silver ion infused lignin-based EbNPs with positive surface charge consist of: (1) a biodegradable EbNP core (negatively charged IAT EbNPs); (2) an active agent (antimicrobial silver ions adsorb on the negatively charged EbNP
25 core); and (3) a surface modifier (polydiallyldimethylammonium chloride [PDADMAC], a positively charged polyelectrolyte). Both systems can attach to negatively-charged bacteria cells. Both systems can release silver ions, which perform the desired antimicrobial function leading to bacteria cell death.

When examining the silver release from the AgNP system, first metallic silver has to
30 dissolve before it can be released in its ionic form into the bacteria. As the change of state from metallic to ionic silver may limit the rate at which silver ions are transferred from the AgNP system to the cell, this may reduce, overall, the antimicrobial efficiency of the system. In contrast to the metallic silver in AgNPs, ionic silver is already available in Ag-EbNPs-PDADMAC at contact with the cell. Therefore, Ag-EbNPs-PDADMAC may be capable of releasing silver ions

more readily, resulting in high antimicrobial efficiency and rapid Ag depletion of the Ag-EbNPs-PDADMAC system. At the end of the lifecycle, both systems may eventually be released into the environment as nanomaterial waste. Moreover, as AgNPs may stay active, releasing reactive silver ions post-utilization, they represent persistent nanoparticle waste that could result in hazards for humans and the environment. On the other hand, the Ag-EbNPs-PDADMAC system, which is depleted of silver ions, is rendered inactive and will degrade over time; hence, Ag-EbNPs-PDADMAC may increase post-utilization safety for humans and the environment.

The article "a" and "an" are used herein to refer to one or more than one (i.e., to at least one) of the grammatical object(s) of the article. By way of example, "an element" means one or more elements.

Throughout the specification the word "comprising," or variations such as "comprises" or "comprising," will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps. The present invention may suitably "comprise", "consist of", or "consist essentially of", the steps, elements, and/or reagents described in the claims.

As depicted in Figure 1, in contrast to permanent nanoparticle systems exhibiting post utilization hazards for humans and the environment, due to nanoparticle migration, accumulation, and persistent activity, the object of the present invention - functionalized EbNPs – have increased nanomaterial safety as they will biodegrade after their intended use minimizing any hazardous effects stemming from nanomaterial waste. Means of synthesizing native EbNPs from biopolymers via green synthesis methods in simple, inexpensive, and non-toxic ways are illustrated. Functionalization options via adsorption of and/or infusion with active agents are described. A range of procedures to alter the EbNP pH stability, colloidal stability in water or organic media, and surface properties have been developed. Silver-infused EbNPs with positive surface charge capable of releasing antimicrobial silver ions were synthesized, and evidence for complete functional equivalency to high-volume nanoparticles currently employed in industry – antimicrobial silver nanoparticles (AgNPs) - was demonstrated. In biocidal tests on human pathogens such as *E. coli* BL21(DE3) and *Pseudomonas aeruginosa*, the EbNPs showed higher efficiency in comparison to AgNPs, and silver nitrate (AgNO₃) solution. While the native EbNPs did not exhibit any observable biocidal activity, positively charged EbNPs infused with silver ions demonstrated significantly higher antimicrobial efficiency. In addition to the beneficial performance with less active agent favoring substitution of AgNPs and others with EbNPs with

functional equivalency, the benign nature of the invention opens opportunities for new applications in safety sensitive applications including the food and drug industry.

The invention can be used as a platform technology for versatile synthesis of functionalized EbNPs, in achieving functionality and efficiency, in formulation, and in applications, and environmental safety and biodegradability at the same time. These EbNPs are synthesized from natural materials available in abundance, potentially from waste or bio-products streams, by employing simple processes using green or no organic solvents. In addition to their safety, efficiency, and cost advantages, the benign nature of the new EbNPs opens multiple utilization opportunities in markets closed for persistent nanoparticles. The advantageous features of the invention include:

- Process novelty: The synthesis procedures for persistent nanoparticle systems often involve hazardous materials, complex methods, and reactions at elevated or reduced temperatures, but only yield small output volumes/yields. Functionalized environmentally benign nanoparticles (EbNPs) are (1) made of sustainable materials and synthesized via novel, green, simple, and scalable methods, (2) functionalized with active agent, and (3) surface modified to achieve complete functional equivalency. Large yields and unmatched cost advantage in comparison to a variety of permanent and hazardous metal or semiconductor nanoparticles can be achieved.
- Functionality and Efficiency: Nanoparticles made entirely of active agent may not make efficient use of the agent. Functionalized EbNPs are primarily made of sustainable materials. The EbNP surface-active functionalization agent can be released more easily increasing the efficiency of the agent. Surface property modification e.g. to protect the active agent, to alter the release properties of the active agent, to change the stability of the system, to alter attractive or repulsive forces to a specific target, to change the charge or hydrophobicity of the system, and to prepare the system for further functionalization and/or modification serve as further possibilities to optimize the efficiency of the EbNPs. As a result, functionalized EbNPs use in general significantly smaller amounts of active agent to deliver the same functionality than their persistent counterparts.
- Additional Safety: Hazardous risks associated with persistent nanoparticle waste have been recognized by the Environmental Protection Agency (EPA) (Hassellöv et al., Nanoparticle analysis and characterization methodologies in environmental risk assessment of engineered nanoparticles. *Ecotoxicology* **2008**, *17* (5), 344-361; Luoma, S. N., Silver nanotechnologies and the Environment. *Woodrow Wilson International Center for Scholars. Washington, DC, USA* **2008**, 72). Other than persistent nanoparticles

exhibiting post utilization hazards for humans and the environment, due to nanoparticle migration, accumulation, and activity, functionalized EbNPs increase nanomaterial safety as they will deplete rendering the particles benign, which biodegrade after their intended use minimizing any hazardous effects stemming from nanomaterial waste.

- Formulation novelty: Choice of materials in functionalized EbNPs results in specific novel ingredient formulations.
- Application novelty: Persistent nanoparticle systems have limited application potential. The benign nature (by design) of the invention opens usage opportunities in markets closed for persistent nanoparticles, which can be found in the pesticide, food, and drug industries.

Functionalized EbNPs comprising, consisting essentially of, or consisting of, a core, one or more functionalizing agents, and one or more surface modifiers, whose details are outlined in the definition of terms, could be based on the following combination of materials according to some embodiments. The EbNP core consists mainly of biopolymers or a combination of biopolymers from the group of modified lignin, modified cellulose, hemi or lingo cellulose, modified chitosan, modified chitin, prolamines, and gluten. Most preferable lignins are modified lignins extracted via Kraft pulping process including sulfonated (Indulin C) and unsulfonated lignin (Indulin AT) from MeadWestvaco and others, and extracted via organosolv process such as High Purified Lignin (HP-L™) from Lignol. Preferable modified lignins include Kraft pulping lignin extracted via Lignoboost process from Metso, and their derivatives. Most preferable modified celluloses include hydroxyl propyl methyl cellulose phthalate (e.g., HP-55 or HP-55S from Shin-Etsu), hypromellose acetate succinate (e.g., AS-HF from Shin-Etsu), and their derivatives. Other preferable modified celluloses include methyl cellulose, ethyl cellulose, cellulose acetate, hydroxyethyl cellulose, cellulose nitrate, cellulose propionate, and their derivatives. Most preferable modified chitosans include low molecular weight (Mw) chitosans, and their derivatives including chemically modified chitosan with amino groups in the backbone of chitin. Preferable chitosans include medium and high Mw chitosan and their derivatives.

The active agent may be any biologically active component. This includes most preferably monovalent and divalent cationic metal ions including biocidal Ag^+ and Cu^{2+} , biocidal semiconductor compounds such as ZnO and TiO_2 , and natural and synthetic pesticides. Preferable active agents are amines, biopolymers with biocide activity including low Mw chitosan with amino groups, and synthetic polymers including negatively and positively charged polyelectrolytes. Other possible functionalization agents include the group of hydrophilic and

hydrophobic pharmaceuticals, food additives, proteins, peptides, and others. Surface property modifiers are surfactants that include synthetic polymers and biopolymers as defined previously. Most preferably are positively charged biocidal amines such as polydiallyldimethylammonium chloride (PDADMAC) or polyallylamine hydrochloride (PAH), and oils and silicon based surfactants used as pesticide transfer and targeting agents. Other groups of surface modifiers include positively and negatively charged polyelectrolytes for surface charge modification, non-ionic surfactants, protein based surfactants, emulsifiers, and polysaccharides.

Negatively charged hydrophobic EbNPs are synthesized via one of the four suggested green synthesis routes with mean diameters most preferably in the range of 20 to 100 nm. Preferable EbNPs from synthesis may also result in bigger particles with mean diameters typically up to 500 nm, or more. The procedures include the water-water based pH-drop method, the solvent-water based pH-drop method, solvent-antisolvent method, and the polyelectrolyte-addition method. Taking lignin as input material, Indulin AT (IAT) and HP-L™ nanoparticles have been synthesized by the previously mentioned procedures.

Table 1 compares the advantages and limitations of each method. In the water-water based pH-drop method, supersaturation and subsequent nanoparticle formation are achieved upon addition of acid to dissolved lignin in water at elevated pH to drop the pH into the range of pH 1.5 to 3.0. The pH stability can be increased upon adsorption of positively charged polyelectrolytes most preferably with PDADMAC, PAH, and others on the negatively charged EbNP surface. In the organic solvent-water based pH drop method, lignin is first dissolved in organic solvent such as ethylene glycol, toluene, or similar. Supersaturation is reached upon addition of acid precipitating out negatively charged hydrophobic EbNPs. The EbNPs formed in organic media may be transferred into water via dialysis or dilution. In the solvent-antisolvent method, biopolymer is dissolved in solvent such as acetone or ethanol. Supersaturation is reached upon rapid addition of antisolvent such as water precipitating out negatively charged hydrophobic EbNPs. In the polyelectrolyte-addition method, biopolymer is dissolved in solvent, typically water at adjusted pH. EbNPs are formed upon addition of positively charged polyelectrolytes.

Table 1: **Green synthesis methods comparison table.**

Method	EbNP material	d [nm]	pH-stability	Main parameters for particle size control	Advantage	Limitations
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water-water pH-drop	IAT, HP-L™	20- 200(+)	1.8 - 3.0	- increasing pH- drop magnitude results in bigger particles - higher initial wt% results in bigger particles	- completely water based	- size control at target pH - pH stability (can be overcome) - native EbNPs not suitable for infusion with active agent
organic solvent-water pH-drop	IAT	20- 500	3.0 - 10.0	- increasing pH- drop magnitude results in bigger particles - higher initial wt% results in bigger particles	- pH stability - size control - physical adsorption of cations possible	- organic solvent residues (can be minimized relatively easy)
solvent- antisolvent	HP-L™	20- 200(+)	3.0 - 10.0	- increasing dilution rate with antisolvent results in smaller particles - higher initial wt% results in bigger particles	- pH stability - size control	- organic solvent residues (can be minimized) - physical adsorption of cations limited
polyelectrolyte addition	IAT, HP-L™	20- 200(+)	3.0 - 10.0	- ratio lignin to polyelectrolyte influences particle size and surface charge	- completely water based	- native EbNPs not suitable for infusion with active agent

Each of the four green synthesis routes is performed at room temperature without crosslinking reaction. This differs significantly from the methods reported in patent literature in which chemically modified cross-linked lignin nanoparticles were synthesized at elevated temperatures (S., M. D. Submicron lignin dispersions. 4957557, 1990; Peter, S. Submicron lignin-based binders for water-based black ink formulations. 5192361, 1993). While chemically modified lignin nanoparticles may not biodegrade as easily, other advantages of the green synthesis methods described include utilization of inexpensive materials, low hazard potential, room temperature operations and therefore no need for external energy input or cooling, size control, scalability, and short synthesis times from prepared stock solutions to synthesized EbNPs in the minute range. According to the advantages outlined, the EbNP synthesis costs are low.

The second part of the invention includes nanoparticle functionalization in order to infuse the matrix with an active ingredient or otherwise create the desired usage characteristics. Functionalization methods of the EbNP carrier include infusion, and absorption and/or physical and chemical adsorption of active agent. Both weak and strong binding of the active agent are possible mechanisms involved in the functionalization. This binding of the agent can occur because of electrostatic interaction, hydrophobic or hydrophilic interactions, reduction processes,

chemical linking, kinetic and entropy driven capture of the functional molecules. In comparison to persistent nanoparticle systems compromised of the active agent alone, the functionalized EbNP technology suggests higher efficiency in terms of optimized smaller amount of active agent used to deliver the same functionality ultimately minimizing risks and hazards stemming from excess active agent.

The adjustment and customization of the surface properties is used to replicate and enhance the particle properties needed for its functionality, and can be achieved by introducing one or more modifiers on the EbNP surface. Depending on the surface modifier chosen, the binding strength and the adsorption processes can vary accordingly. Surface properties that are controlled on this stage include surface charge, pH stability, hydrophobicity, biocidal activity, and others. Changes in surface properties can specifically be performed for better particle targeting, to increase the shelf life of the system, to alter the colloidal stability, to modify the interaction potential with the environment or a specific target, to protect the active agent, to customize depletion and transport effects of active agent, and others.

Applications:

The EbNP systems taught herein can find applications in different areas of technology and industrial products. The functionalized EbNPs may be designed to exhibit locally confined and temporarily limited bioactivity, by delivering the same desired activity as permanent nanoparticles currently employed in various applications, but only during the time of their application. Since functionalized EbNPs can be engineered to have complete functional equivalency to a variety of permanent hazardous nanoparticles, EbNPs may therefore replace a wide range of metal or semiconductor nanoparticles employed at moderate temperatures.

The benign nature of the invention opens opportunities for its use in markets presently closed for persistent nanoparticles. New additional applications may be found in the pesticide, food, and drug industries. Applications of functionalized EbNPs are sectioned in immediate applications, new applications, and new applications with FDA approved EbNP matrix.

Applications of these new particles include / invention can be employed as:

- a) Functionalized environmentally benign nanoparticles (EbNPs) with antimicrobial properties to be used as an additive to detergents or soaps to establish or increase the antimicrobial, antifungal, or antiviral function of the product.
- b) Surface coatings made of biodegradable components consisting of modified lignin and/or modified cellulose, hemicellulose or chitin functionalized with positively charged

polyelectrolytes and/or Ag⁺ ions adsorbed in/on the matrix for antimicrobial, antifungal, and antiviral surface functionalization in consumer products.

- 5 c) Functionalized EbNP suspensions for dipping or spraying with added modified lignin and/or modified cellulose, hemicellulose or chitin in diameters ranging from 20 to 500 nm, optionally functionalized with positively charged polyelectrolytes, and/or Ag ions adsorbed on the particles, in water-based or organic solvent-based solution, for antimicrobial, antifungal, and antiviral functionalization of surfaces.
- 10 d) Functionalized EbNPs comprising, consistent essentially of, or consisting of, modified lignin and/or modified cellulose, hemicellulose or chitin in diameters ranging from 20 to 500 nm functionalized with positively charged polyelectrolytes and/or Ag ions adsorbed on the particles to be incorporated in/ adsorbed on general available water filtration matrixes to provide antimicrobial, antifungal, and antiviral water treatment in addition to basic water filtration.
- 15 e) EbNPs consisting of modified lignin and/or modified cellulose, hemicellulose or chitin in diameters ranging from 20 to 500 nm functionalized with biocidal agents such as Ag⁺, Cu²⁺, or iron oxides coated with positively charged polyelectrolyte incorporated in/ adsorbed on general available water filtration matrixes to provide antimicrobial, antifungal, and antiviral water treatment in addition to basic water filtration.
- 20 f) Functionalized EbNPs to be used as general active agent carrier system specifically to substitute silicon based particle systems used in the pesticide and food industry.
- g) EbNPs functionalized with pesticides and coated with pesticide transfer agent to be used as benign pesticide agent carrier and delivery system with engineered temporal activity to reduce the amount of active agent used and therefore any negative environmental impact while delivering the same functionality, and to increase product safety for humans.
- 25 h) Functionalized EbNPs in diameters ranging from 20 to 500 nm functionalized with positively charged polyelectrolytes and/or Ag⁺ ions adsorbed on the particles to be incorporated in and/or adsorbed on general types of water filtration matrixes to provide antimicrobial, antifungal, and antiviral water treatment in the pharmaceutical industry.
- 30 i) Nano-carriers made of biopolymers in diameters ranging from 20 to 500 nm functionalized with positively charged polyelectrolytes and/or Ag⁺ ions adsorbed on the particles to be incorporated in and/or adsorbed on general types of water filtration matrixes to provide antimicrobial, antifungal, and antiviral water treatment in the food industry.

- j) EbNPs in diameters ranging from 20 to 500 nm functionalized with biocidal agents such as Ag^+ , Cu^{2+} , or iron oxides coated with positively charged polyelectrolyte incorporated in/ adsorbed on general available water filtration matrixes to provide antimicrobial, antifungal, and antiviral water treatment in addition to basic water filtration.
- 5 k) EbNPs functionalized with active agent to be used as general active agent carrier system specifically to substitute silicone-based particle systems used in the pesticide industry.
- l) EbNPs functionalized with pesticides and coated with pesticide transfer agent to be used as benign pesticide agent carrier and delivery system with engineered temporal activity to reduce the amount of active agent used and therefore any negative environmental impact while delivering the same functionality, and to increase product safety for humans.
- 10 m) EbNPs functionalized with organic pesticides and coated with pesticide transfer agent to be used as benign pesticide agent carrier and delivery system with engineered temporal activity to reduce the amount of active agent used and therefore any negative environmental impact while delivering the same functionality, and to increase product safety for humans.
- 15 n) EbNPs functionalized with antimicrobial agent to be used as benign antimicrobial agent carrier and delivery system with engineered temporal activity to combat bacterial and fungal plant pathogens to reduce vegetable spoilage.
- o) EbNPs functionalized with antimicrobial agent to be used to functionalize dressings and tampons with antimicrobial effect to decrease the potential of infections.
- 20 p) EbNPs functionalized with antimicrobial agent to be used to functionalize an article of hygiene with antimicrobial properties to decrease the potential of infections.
- q) EbNPs functionalized with antimicrobial agent to be used to functionalize paper with antimicrobial properties to decrease the potential of infections.

25 New applications include:

- r) EbNPs comprising, consistent essentially of, or consisting of, modified lignin and/or modified cellulose, hemicellulose or chitin in diameters ranging from 20 to 500 nm, optionally functionalized with positively charged polyelectrolyte, to be used as adsorption matrix for monovalent and divalent hazardous metal ions and hydrophobic agents in liquid-based environmental remediation processes.
- 30 s) Process of making EbNPs comprising, consistent essentially of, or consisting of, modified lignin synthesized via solvent - antisolvent (where water could be both solvent and anti-solvent) process from organosolv lignin, or synthesized via pH drop method in

organic solvent or water, or synthesized via addition of negatively charged polyelectrolyte to lignin dissolved in solvent, and/or modified cellulose synthesized via pH drop method in organic solvent or water, or synthesized via addition of negatively charged polyelectrolyte to cellulose dissolved in solvent, in diameters ranging from 20 to 500 nm, optionally functionalized with positively or negatively charged polyelectrolyte, to be used as environmentally benign fillers and protectors in paints in the micro and nano size range in wood constructions.

- t) EbNPs functionalized with biocides to be used as paint additive to decrease susceptibility of growth of prokaryotic or eukaryotic cells on the surface.
- u) EbNPs functionalized with biocides to be used as additives in sound or heat isolation materials or fillers to decrease susceptibility of growth of prokaryotic or eukaryotic cells on the surface or in the bulk of such materials.
- v) Hydrophobic EbNPs with optional biocidal functionalization to be used in wood protection and/or as paint additive to decrease both the wettability of the product and the growth of prokaryotic and eukaryotic cells on the surface of wood products to increase their lifetime.
- w) EbNPs to be used as binder additive into paints, ceramics, glues, or coating materials dispersed in the product to serve as linker between the components upon drying increasing the stability of the product.
- x) Functionalized EbNPs to be gathered in porous particles frits to serve as catalyst facilitating chemical reactions.
- y) EbNPs functionalized with catalytic metals such as Pt or Pt alloys immobilized in porous frits to serve as catalysts facilitating chemical reactions.
- z) Functionalized EbNPs with surface coating with specific adsorption potential to serve as adsorption material for targeted biomolecules to be used in biosensor applications.
- aa) Bioassays using EbNPs with possibly additional marker or dye functionalization with surface coating exhibiting adsorption potential to specific biopolymers to be used in biomolecular screening applications.
- bb) Functionalized EbNPs to be used on bioassay microchip to serve as biomolecule specific adsorption material.
- cc) Functionalized EbNPs comprising, consistent essentially of, or consisting of, synthesized EbNPs and at least one polyelectrolyte to be used as coating material to alter surface properties including hydrophobicity, surface charge, and optional anisotropy.

dd) Foam or emulsion based products, where bubbles or droplets are fully or partially stabilized with EbNPs, having at least one of the additional functionalities as described above, where the additional functionality of the particles is boosted (in terms of dose or efficiency) due to their attachment of the interface and their benign properties further improved when these foams or emulsions are destabilized.

New therapeutic or nutraceutical applications (possibly for FDA-approved or other regulatory approval) of EbNPs described herein include:

ee) Functionalized EbNPs with biocide functionalization to be employed as coating material in food containers, bottles, or cans to decrease antimicrobial or antifungal contamination potential to protect the food from subsequent spoiling.

ff) Functionalized EbNPs with biocide functionalization to be used as food additive to increase the shelf life through making the food less susceptible for bacterial or fungal contamination, and to decrease spreading of bacteria and fungi in the product.

gg) Functionalized environmentally benign nanoparticles consisting of modified lignin and/or modified cellulose, hemicellulose or chitin in diameters ranging from 20 to 500 nm to adsorb hydrophobic drugs and beneficial cationic ions and molecules prior to optional protective functionalization with positively and negatively charged polyelectrolytes to be used as transport agent in oral drug delivery.

hh) Functionalized EbNPs with antimicrobial properties added as active agent in disinfecting and cleaning solutions for soft and rigid contact lenses to protect the eyes of the contact lens wearer from infections.

ii) Functionalized EbNPs with insecticide, herbicide, or fungicide functionalization to be employed as pesticide in the agriculture sector to protect the pesticide from dilution and depletion, to specifically target the pest, and to increase the pesticide concentration locally at the target while decreasing the overall average exposure of pesticide on the protected good.

jj) EbNPs with marker or dye functionalization to be employed as a diagnostic or an indicator for exposure routes in humans, animals, and the environment in general to protect the marker from dilution and depletion, to decrease the overall indicator concentration while using a benign matrix.

kk) EbNPs functionalized with antibiotics to be delivered in humans, animals, and the environment in general to protect the agent from dilution, depletion, and degradation, to decrease the overall antibiotic exposure concentration while using a benign matrix.

- ll) EbNPs with Ag infusion to be used as wound dressing, and immobilized antibacterial, antifungal, anti-inflammatory active agent on material used as wound cover.
- mm) EbNPs with Ag infusion to be incorporated in hydrogels to provide wound dressing with antibacterial, antifungal, and anti-inflammatory properties.
- 5 nn) Functionalized EbNPs with antimicrobial agents to be used as coating materials of catheters including catheters used in neurosurgery for cerebrospinal fluid drainage to reduce the risk of infection.
- oo) Functionalized EbNPs with antimicrobial agents to be used as additive to bone cement and implantable materials to reduce risks of infections.
- 10 pp) Ag functionalized EbNPs with beneficial properties towards treatment of dermatitis to be used as in cures resulting in reduction of dermatitis due to Ag ions stemming from EbNP system.
- qq) Ag functionalized EbNPs with beneficial properties towards treatment of acne resulting to be used in acne treatment solutions in reduction of acne due to Ag ions stemming from
- 15 EbNP system.
- rr) Functionalized EbNPs with antimicrobial properties to be used in urology as coating material for surgical mesh to provide protection towards bacterial infections for pelvic reconstruction.
- ss) Functionalized EbNPs (in one non-limiting embodiment, Ag^+) with antiviral properties to
- 20 be used as virus replication inhibitor in direct virus treatments, and in vaccines.
- tt) EbNPs functionalized with bactericidal and antifungal agents to be used as preservatives in vaccines to protect the vaccine agent from contamination and therefore, the person vaccinated from infections related to contaminations.
- uu) EbNPs functionalized with bactericidal and antifungal agents to be used as coating
- 25 material in vials, vessels, or syringes holding vaccines to protect the vaccine agent from contamination and therefore, the person vaccinated from infections related to contaminations.

Definition of terms

- 30 - Biopolymers include linear, branched, or cross-linked biopolymers of natural or technical origin (i.e. physically and/or chemically modified during processing of natural compounds and appearing usually as bi-product or waste stream of such process)
- Biopolymers include large molecular weight natural or technical polyphenols or natural phenolic polymers like lignin or modified lignin or a combination thereof.

- Biopolymers from natural or technical origin include physically and/or chemically modified biopolymers during processing of natural compounds that appear usually as bi-product or waste stream of such process.
- Biopolymers include byproducts of degradation of lignin, such as humic acid and others.
- 5 - Organic solvents include acetone, ethylene glycol, propylene glycol, glycerol, methyl acetate, ethyl acetate, methanol, ethanol, hexanol, petrol ether, toluene, benzene, turpentine, dichlormethane, chloroform, formic acid, isopropanol, polyethylene oxide, and others.
- Prolamines include gliadin from wheat, hordein from barley, secalin from rye, zein from
10 corn, kafirin from sorghum or avenin from oats.
- Gluten include gliadin and glutenin.
- Cationic metal ions include Ag^+ , Ag^{2+} , Cu^{2+} , Zn^{2+} , Ni^{2+} , Co^{2+} , Fe^{2+} , others, and their complexes; precursor of metal ions for e.g., Ag^+ are salts such as AgNO_3 .
- Anionic ions include anionic antimicrobial peptides and proteins (AAMPs).
- 15 - Cytotoxic agents include but are not limited to antibacterial agents such as antibacterial peptides or proteins. Examples include, but are not limited to, bactericidal/permeability-increasing protein (BPI); helical linear peptides, e.g., gramicidin, magainins, melittin; or β -sheet or cyclic peptides such as defensins, amphotericins and nystatin.
- Semiconductors include ZnO, ZnSe, ZnS, ZnTe, CuCl, Cu_2S , CuO, TiO_2 , CdSe, CdS,
20 CdTe FeO, Fe_3O_4 , (α , β , γ , ϵ), Fe_2O_3 , Fe_2S , NiO, Ag_2S , and others.
- Pesticides include algaecides, avicides, bactericides, fungicides, insecticides, miticides, molluscicides, nematocides, rodenticides, glyphosate, and virucides.
- Amines include primary, secondary, tertiary, and quarternary structures. This includes polydiallyldimethylammonium chloride (PDADMAC), poyallylamine hydrochloride
25 (PAH), polyethyleneimine (PEI), branched polyethyleneimine (BPEI), polyethoxylated tallow amine (POEA), and others.
- Non-ionic surfactants include alcohol ethoxylates, alcohol ethoxyfulfates, and others.
- Silicon based surfactants include silicone and silicone blends with polysiloxane chains, and include commercial products such as Sylgard[®] 309 (Wilbur-Ellis Company),
30 Freeway[®] (Loveland Industries), Dyne-Amic[®] (Helena Chemical Company), and Silwet L-77[®] (Loveland and Helena), and others.
- Oils include vegetable oil, methylated vegetable oil, seed oils, crop oils, petroleum based oils, silicon based oils, and blends of these. Commercial products include MSO[®] Concentrate Methylated Seed Oil (Loveland Industries), Hasten[®] (Wilbur-Ellis

Company), Improved JLB Oil Plus (Brewer International), Cide-Kick and Cide-Kick II (Brewer International), Syl-tac™ (Wilbur-Ellis Company), Phase™ (Loveland Industries), Agri-dex® (Helena Chemical Co. or Setre Chemical Co.), Red-Top Mor-Act® (Wilbur-Ellis Company), and others.

- 5 - Other surface modification and activation agents include amphiphilic proteins, soy proteins, proteins, DNA, fluorescence DNA, peptides, fluorescence markers, amino acids, and others.

10 The Examples presented herein further illustrate the invention and are not intended to limit the scope of the invention.

EXAMPLES

Example 1

15 Here, we report non-limiting, exemplary data on the synthesis of native HPL and IAT EbNPs, the infusion of native IAT EbNPs with silver ions, the surface charge modification of the system with PDADMAC, and the quantification of antimicrobial efficiencies for opportunistic human pathogens *E. coli* and *P. aeruginosa*. In addition, and though not wishing to be bound by theory, we provide a hypothesis to explain the antimicrobial mechanism associated with Ag-
20 EbNPs-PDADMAC.

1 RESULTS

1.1 *Synthesis and characterization of HPL EbNPs*

25 We synthesized native HPL EbNPs via the solvent-antisolvent precipitation method. As illustrated in Figure 2, hydrophobic HPL is first dissolved in a good solvent, which is acetone, and rapidly transferred into an antisolvent, which is Millipore H₂O. Upon change of media, HPL may precipitate out as stable negatively charged EbNPs. The main parameters controlling the size and size distribution of these EbNPs include the initial HPL loading in the solvent, and the rate of dilution with antisolvent.

30 The data on the effect of the initial HPL loading in the solvent on the final EbNP size are shown in Figure 3. The TEM images may indicate spherical particles with diameters below 100 nm. The preparation of the samples included the following two steps (1) 1 ml of HPL dissolved in acetone was placed in a 20 ml scintillation vial, (2) 9.21 ml of Millipore

H₂O was rapidly added to the solution immediately precipitating out HPL EbNPs. The samples obtained were then further diluted to 0.05 wt%, which is a suitable concentration for size measurements with dynamic light scattering (DLS). The EbNP z-average diameters increased with increasing amount of HPL in the initial solvent. Repeated testing, as indicated with error bars, shows that the target diameters are reproducible. While the polydispersity width increases with particle size, the polydispersity indexes, which fluctuate mainly between 0.05 and 0.20, did not show a distinct trend. The wide polydispersity width observed could be a consequence of the broad molecular weight size distribution of the raw material HPL. The stability of the particle suspensions over time was confirmed with a size measurement performed after 84 days, which did not show any significant change in diameter.

1.1.1 *HPL EbNP pH stability*

The stability of HPL EbNPs against pH change was tested in dialyzed 0.10 wt% HPL EbNP samples, which were diluted down to 0.05 wt%. To adjust the pH of the EbNP suspensions, calibrated amounts of HNO₃ or NaOH solution were added. As indicated in Figure 4, the pH stability of the EbNPs ranges from 3.25 to 10. Below a pH value of 3.25, the EbNPs show signs of instability and eventually aggregate as indicated with the threefold size increase at pH 2.91, which is accompanied with an apparent ζ -potential drop. When investigating the samples above pH 10, we observed a color change accompanied with reduced light scattering intensity, which was confirmed by a vanishing count rate at the DLS, indicating dissolution of HPL EbNPs.

1.2 *Synthesis and characterization of IAT EbNPs*

Figure 5 illustrates two IAT EbNP synthesis protocols. IAT EbNPs synthesized in ethylene glycol exhibit pH stability due to favourable stacking of the IAT molecules, while IAT EbNPs synthesized via the water-based pH drop method dissolve upon pH increase. Figure 6 shows the diameters of IAT EbNPs synthesized via the water-based pH-drop method as a function of acid added to the system. Here, we added rapidly under vigorous stirring defined amounts of HNO₃ to 10 ml of 0.05 wt% IAT solution at pH 12 inducing a sudden pH drop, which triggered IAT precipitation as EbNPs. The final pH values of the samples are reported on the top x-axis. Size control could be achieved by appropriately adjusting the amount of acid added.

Native IAT EbNPs were synthesized via the organic solvent water-based pH-drop method in ethylene glycol and the data are reported in Figure 7. This method allows the synthesis of IAT EbNPs with good size control in the range of 50 to 125 nm. First, 0.25 g commercial IAT lignin was dissolved in 50 ml of ethylene glycol, vortexed for 30 minutes, and filtered with a 0.45 μ m syringe filter. Then, 5 ml of the stock solution was put into a 20 ml scintillation vial and vigorously stirred with a fitting magnetic stir bar. Supersaturation was induced upon addition of various amounts of acid precipitating out negatively charged IAT EbNPs. We diluted the EbNP suspension in ethylene glycol with Millipore H₂O, after 5 minutes and 7 days, to obtain a 0.05 wt% IAT EbNP sample, containing 10% (v/v) of ethylene glycol. The pH values measured for these final suspensions range from 3 to 4. The EbNPs exhibited a negative ζ -potential with a magnitude of approximately - 30 mV for all samples. The polydispersity widths increase slightly with increasing particle diameters. The 7 day synthesis shows that the particles are growing when kept in ethylene glycol solution. The IAT EbNPs dispersed in water are stable for periods longer than 84 days.

1.2.1 IAT EbNP pH stability

As reported in Figure 8, the IAT EbNPs obtained are pH-stable in the pH range from 3 to 9. Here, we took dialyzed native IAT EbNP suspensions at pH 4.5 and adjusted the pH with NaOH or HNO₃ solution. The z-average diameter and the ζ -potential of each sample were measured. The colloidal instability of the IAT EbNP suspensions below pH 3 can be explained with the drop in ζ -potential indicating decreased electrostatic repulsion between the particles. At higher pH values, the decrease in particle size may indicate dissolution of particles. To quantify the dissolution of IAT EbNPs at elevated pH, we performed a UV-Vis study to determine the remaining amount of IAT lignin precipitated as particles as a function of increasing pH. The pH stability of IAT EbNPs is reported in Figure 9. We started with dialyzed IAT EbNPs at pH 4.92 and adjusted the pH value afterwards. We then took the supernatant of these pre-treated samples and added aliquots of NaOH solution to baseline the samples. Then, we determined the IAT lignin dissolved in the supernatant and closed the IAT mass balance to obtain the mass of IAT EbNPs remaining in the suspension at each pH value. For example, when we increase the pH of the native EbNP suspension from pH 4.92 to pH 8.2, 53.8% of the initial IAT EbNPs remain in form of EbNPs, while 46.2% of the initial IAT EbNPs are dissolved.

1.2.2 *IAT EbNP ionic strength study and DLVO modeling*

We performed an ionic strength study to investigate the effect of increasing ionic strength on the stability of IAT EbNP suspensions, and to determine if the IAT EbNP suspensions may exhibit colloidal stability at ionic strength levels equivalent to the ones found in physiological testing media used in biocidal testing. Figure 10 shows the particle diameter and ζ -potential as a function of ionic strength in mol/L. We added measured amounts of NaCl to adjust to the target ionic strength – 0.10 M NaCl is equivalent to 0.10 mol/L ionic strength. The colloidal stability of the EbNP suspension was confirmed by DLS size measurements. The magnitude of the measured ζ -potential decreases rapidly upon a small increase of ionic strength. We observed that the sample at 0.30 mol/L, at an ionic strength well above the one found in physiological testing media (0.015 mol/L), exhibited colloidal stability even after 36 days. The EbNP suspensions above 0.30 M NaCl started to show signs of colloidal instability in the form of settling and aggregation.

We modeled the interaction energy $W(D)$ according to DLVO theory in selected IAT EbNPs at three chosen ionic strengths. At an ionic strength of 0.25 mol/L, we determined a Debye length $k^{-1} = 0.61$ nm. With a ζ -potential of -15.0 mV at that ionic strength, we calculated a surface potential $\Psi_0 = -40.8$ mV. We determined the electrostatic repulsion energy $W(D_{elec})$ and the van der Waals attraction energy $W(D_{VDW})$ as a function of the separation distance D to evaluate the total interaction energy $W(D)$. As shown in Figure 11, the stability threshold in the colloidal system was determined to be at 0.25 mol/L ionic strength. The colloidal suspension at 0.50 mol/L ionic strength shows a highly unstable sample.

1.3 *Characterization of Ag^+ ion infused EbNPs*

We obtained IAT EbNPs with a hydrodynamic diameter of 72 nm with a polydispersity index of 0.230 and a ζ -potential of -23.5 mV. The DLS equipment measured a conductivity of 0.139 mS/cm, and an electrophoretic mobility of -1.403 $\mu\text{m cm}^2/\text{Vs}$. As depicted in Figure 12, TEM micrographs show predominantly nanosized non-spherical clusters in the size range below 100 nm. Some degree of spreading of IAT particles on the TEM grid could be triggered by the hydrophilic nature of IAT. The structures of the clusters suggest a high availability of surface area for particle functionalization.

We functionalized negatively charged IAT EbNPs with Ag^+ ions in aqueous solution. We chose a common soluble salt, $AgNO_3$, as an Ag^+ ion source. Figure 12 illustrates the possible adsorption of Ag^+ ions on the deprotonated ionized groups on the EbNP surface. The main

functional groups of IAT lignin include phenolic –OH, aliphatic –OH, carboxyl groups –OOH, and thiol groups –SH, which when deprotonated render the surface charge of IAT EbNPs negative; hence, deprotonated functional groups serve as suitable binding sites for cations including Ag^+ ions. The distribution of the functional groups and the respective pKa values are reported in Table 6. TEM micrographs of functionalized IAT EbNPs show predominantly nanosized non-spherical clusters in the size range below 100 nm.

We prepared AgNO_3 standards with 40 ppm Ag^+ , 100 ppm Ag^+ , 200 ppm Ag^+ , and 800 ppm Ag^+ , and added 0.5 ml of each of these standards to 9.5 ml of previously prepared 0.0526 wt% IAT EbNP suspensions to infuse the particles with Ag^+ ions. To determine the Ag^+ ion content adsorbed on the EbNPs, we first determined the residual Ag^+ ions in the supernatant in each sample, and then closed the Ag^+ ion balance to estimate the amount of Ag^+ ions adsorbed on the particles. The ion content in the supernatant was determined with an Ag^+ ion selective electrode (ISE).

Table 2 summarizes the Ag^+ ion infused samples with various amounts of Ag^+ ion loadings. The initial loading corresponds to the overall Ag^+ ion content in the 10 ml sample at the time of infusion. The Ag^+ ion content in the supernatant was calculated from the mV reading at the ISE with the following equation.

$$\text{Ag}^+_{\text{snat}}[\text{ppm}] = e^{\left(\frac{\text{ISE}[\text{mV}] + 100.74}{26.105}\right)}$$

We measured a negative ζ -potential for the Ag^+ infused IAT EbNPs.

Table 2: Ag^+ adsorption data on particles.

EbNP wt% , initial Ag^+ ppm loading	Ag^+ ion ISE [mv]	Supernatant [ppm Ag^+]	Adsorbed on particles [ppm Ag^+]	Ag^+ ion adsorbed on particles [%]
IAT 0.05, 2ppm	-98.80	0.91	1.09	54.7
IAT 0.05, 5ppm	-64.80	3.58	1.42	28.4
IAT 0.05, 10ppm	-44.30	7.70	2.30	23.0
IAT 0.05, 40ppm	-5.70	35.07	4.93	12.3

We modeled the Ag^+ ion adsorption equilibria with a Langmuir adsorption isotherm and normalized the Ag^+ uptake capabilities per surface area. The Langmuir adsorption isotherm is described by the following equation:

$$\Gamma(c) = \Gamma_{max} \frac{Kc}{1 + Kc}$$

We determined a maximal adsorption $\Gamma_{max} = 8688 \text{ ppm Ag}^+ / \text{m}^2$ particle surface area, and a K value of 0.000115. We used these parameters to model the adsorption isotherm that we report in Figure 13. We observed that the Ag^+ ion loading increases with an increasing amount of Ag^+ available. The Ag^+ absorption reaches equilibrium within 24 h. We observed that the Ag^+ content in the supernatant stays constant after 24h (re-measured after 3 days). We infer that the Ag^+ is predominately physically adsorbed on the IAT binding sites.

1.4 Synthesis and characterization of Ag-EbNPs-PDADMAC

To allow electrostatic attraction between negatively charged bacteria in aqueous solution and the Ag^+ ion functionalized EbNPs, we reversed the surface charge of Ag^+ infused EbNPs from negative to positive. We modified the surface properties of the EbNP system through adsorption of PDADMAC, a positively charged polyelectrolyte. To find a suitable PDADMAC concentration for the EbNP surface modification, we prepared samples with the initial PDADMAC concentrations reported in Table 3. The particles were coated in 5 ml batches of IAT EbNP 0.05 wt% suspension. For the surface modification step, 5 ml of polyelectrolyte solution with the previously reported wt% was added rapidly to the IAT EbNP suspension. The final concentration of IAT in the sample was 0.025 wt%. To investigate the stability and the change of properties of the coated samples, we measured the z-averages and the ζ -potential with DLS. Figure 14 illustrates the EbNP diameter and ζ -potential trends as a function of initial PDADMAC concentration. The results indicate that below addition of 0.10 wt% PDADMAC solution, the IAT EbNP surface potential does not reverse from negative to positive. At 0.15 wt% or higher, enough PDADMAC is available to reverse the surface charge to positive.

Table 3: Initial PDADMAC wt% and resulting ζ -potential after polyelectrolyte coating.

PDADMAC wt% initial	dispersant) with 5%EG [nm]	ζ -potential [mV]	Comments
0.050	78.07	38.5	Stable

0.025	73.4	36.4	Stable
0.020	73.81	32.9	Stable
0.015	74.65	30.6	Stable
0.010	77.86	23.5	
0.005	69.54	-23.0	
0.001	75.2	-24.3	

Table 4: Initial PAH wt% and resulting ζ -potential after polyelectrolyte coating.

PAH wt% initial	z-average (adjusted dispersant with 5%EG [nm])	zeta potential [mV]	Comments
0.050	79.21	42.8	stable
0.020	76.97	42.1	stable
0.010	86.94	38.6	stable
0.005	18.59	13.8	unstable

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The magnitude of the positive surface potential, obtained after coating the IAT EbnPs, is dependent on the polyelectrolyte used. Similarly to the samples with PDADMAC coating reported previously, polyallylamine hydrochloride (PAH) coated IAT EbnPs were synthesized to prove the possibility to customize the surface charge magnitude by choice of suitable polyelectrolytes. The z-averages and ζ -potentials were measured and are reported in Table 4. The corresponding trends are illustrated in Figure 15.

A suitable sample obtained was Ag-EbnPs-PDADMAC ($d=72$ nm) with a final IAT EbnP concentration of 0.025 wt%, an Ag^+ ion content on the particles of 0.71 ppm, an Ag^+ ion amount in the supernatant of 1.79 ppm, and a PDADMAC concentration of 0.01 wt% in the colloidal suspension. The surface potential was reversed from -25.0 mV to +32.4 mV with the

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addition of PDADMAC – 0.01 wt% in the final sample. The final sample pH was 5.5. Other samples were prepared accordingly.

1.5 Antimicrobial testing

We compared the antimicrobial activity of Ag-EbNPs-PDADMAC with that of positively charged branched polyethylene imine AgNPs (BPEI AgNPs) and AgNO₃ solutions (see supplemental information for BPEI AgNP and AgNO₃ sample preparations). We performed quantitative antimicrobial tests on Gram-negative *E. coli* BL21 (DE3), a common human pathogen, and qualitative tests on Gram-negative *P. aeruginosa*, a human pathogen not susceptible to antimicrobial amines such as BPEI and PDADMAC. Therefore, any antimicrobial activity in the *P. aeruginosa* tests will predominantly stem from silver.

The activity of each active agent was determined by comparing the number of colony forming units (CFU) of a reference plate with the CFU of a test plate as depicted in Figure 16. The reduction of CFU on a test plate with antimicrobial agent is time dependent and concentration dependent.

The maximum antimicrobial reduction efficiency of 100% was reached when no CFU could be determined on the test plate. We quantified by the antimicrobial reduction efficiency "E" with the following equation

$$E = 100 \left(1 - \frac{CFU_{sample}}{CFU_{reference}} \right)$$

The schematic in Figure 17 describes the wet method procedure that was followed for antimicrobial tests on both *E. coli* and *P. aeruginosa*. First, 200 µl of each active agent was placed into separate low retention centrifuge tubes. 100 µl of PBS buffer was added to each tube to baseline the ionic strength, and to adjust the pH value to 7. Finally, 100 µl of bacteria, *E. coli* or *P. aeruginosa* solution with approximately 4400 CFU/ml in nutrient broth, was added. The samples were continuously vortexed. After the bacteria were exposed to the active agent for 1 minute, the survival rate of the bacteria was determined by plating 100 µl of each sample evenly distributed on Luria-Bertani agar plates. The procedure was repeated after 30 minutes of exposure time. After the plating procedure, the petri dishes were sealed and incubated upside-down for 48 h at 37° C.

1.5.1 Quantitative antimicrobial test on *E. Coli*

Figure 18 and Table 5 compare the quantitative antimicrobial efficiency of each active agent in the *E. coli* tests. The reduction efficiency of six different samples with increasing Ag ppm equivalent ranging from 0 ppm to 54 ppm was investigated. The graphs show the reduction efficiency at two time points, 1 minute and 30 minutes. The weight percentages of the control samples and the silver contents in the active agents were chosen to show antimicrobial thresholds and to facilitate comparisons between the samples. Native IAT EbNPs without Ag⁺ functionalization and surface modification did not result in any observable reduction in CFU (not reported), which suggests that the native IAT EbNPs are benign. Also, IAT EbNPs with Ag⁺ functionalization but without PDADMAC coating did not result in significant reduction of CFU after 1 minute (0%) and 30 minutes (5%). We suggest that the low antimicrobial efficiency may be attributed to the negative surface charge of these EbNPs, which may hinder them from overcoming the electrostatic barrier between the particles and the bacteria. IAT EbNPs coated with PDADMAC resulted in strong reduction of CFU after an exposure time of 30 minutes, which may be attributed to the antimicrobial effect of the quaternary amine PDADMAC. PDADMAC solution alone (not reported) exhibited strong bactericidal effects towards *E. coli* as well, comparable to the efficiency of Ag-EbNPs-PDADMAC or 100% after 30 minutes exposure time. Ag-EbNPs-PDADMAC exhibited strong reduction in CFU, prevalent after 1 minute exposure time. The corresponding supernatant of Ag-EbNPs-PDADMAC exhibited no observable effect after 1 minute. The reduction of CFU in the supernatant after 30 minutes exposure time may be explained by residue active agent in the solution. BPEI AgNPs and AgNO₃ solutions exhibited antimicrobial effects at 20 ppm Ag and 40 ppm Ag respectively. Overall, the Ag-EbNPs-PDADMAC sample outperformed the BPEI AgNPs and AgNO₃ samples in terms of antimicrobial efficiency normalized on Ag ppm equivalent.

Table 5: Results of quantitative *E. coli* tests. The CFU reduction efficiency of selected IAT EbNP samples, BPEI AgNP samples, and AgNO₃ samples are shown.

Quantitative <i>E. coli</i> test; IAT EbNP samples
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1min vortex time	IAT0.05 Ag ⁺ 5ppm	IAT0.025 Ag ⁺ 2.5ppm PDADMAC 0.01	IAT0.025 PDADMAC 0.01	snat IAT0.025 Ag ⁺ 2.5ppm PDADMAC 0.01	
CFU reduction efficiency	<i>no observable effect</i>	95.24%	12.38%	<i>no observable effect</i>	
30 min vortex time	IAT0.05 Ag ⁺ 5ppm	IAT0.025 Ag ⁺ 2.5ppm PDADMAC 0.01	IAT0.025 PDADMAC 0.01	snat IAT0.025 Ag ⁺ 2.5ppm PDADMAC 0.01	
CFU reduction efficiency	4.76%	100.00%	100.00%	89.52%	
Quantitative <i>E. coli</i> test; AgNPs					
1min vortex time	BPEI AgNPs 54ppm	BPEI AgNPs 20ppm	BPEI AgNPs 10ppm	BPEI AgNPs 5ppm	BPEI AgNPs 2.5ppm
CFU reduction efficiency	84.76%	<i>no observable effect</i>	<i>no observable effect</i>	<i>no observable effect</i>	<i>no observable effect</i>
30 min vortex time	BPEI AgNPs 54ppm	BPEI AgNPs 20ppm	BPEI AgNPs 10ppm	BPEI AgNPs 5ppm	BPEI AgNPs 2.5ppm
CFU reduction efficiency	100.00%	91.43%	<i>no observable effect</i>	<i>no observable effect</i>	<i>no observable effect</i>
Quantitative <i>E. coli</i> test; AgNO ₃					
1min vortex time	AgNO ₃ 40ppm	AgNO ₃ 20ppm	AgNO ₃ 10ppm	AgNO ₃ 5ppm	AgNO ₃ 2.5ppm
CFU reduction efficiency	53.33%	<i>no observable effect</i>	<i>no observable effect</i>	<i>no observable effect</i>	<i>no observable effect</i>
30 min vortex time	AgNO ₃ 40ppm	AgNO ₃ 20ppm	AgNO ₃ 10ppm	AgNO ₃ 5ppm	AgNO ₃ 2.5ppm
CFU reduction efficiency	96.19%	<i>no observable effect</i>	<i>no observable effect</i>	<i>no observable effect</i>	<i>no observable effect</i>

1.5.2 Qualitative antimicrobial test on *P. aeruginosa*

As mentioned previously, the qualitative antimicrobial test on *P. aeruginosa* can distinguish the antimicrobial effect of PDADMAC from the effect of silver. Figure 19 and Table 6 compare the qualitative antimicrobial efficiency of each active agent in this test. BPEI AgNPs and AgNO₃ solutions exhibited no complete antimicrobial effect after 30 minutes incubation time at 54 ppm Ag and 20 ppm Ag respectively. The control sample IAT EbNP at an elevated wt% of 0.10 did not result in any observable effect. Also, the sample IAT EbNPs coated with PDADMAC did not exhibit any measureable antimicrobial effect. The PDADMAC solutions at 0.02 wt% and 0.04 wt% appeared to promote *P. aeruginosa* growth. The sample Ag-EbNPs-PDADMAC was the only one that exhibited complete or 100% antimicrobial efficiency after 30 minutes. The supernatant of the sample Ag-EbNPs-PDADMAC did not show any antimicrobial effect after 30 minutes of incubation time.

Table 6: Result table of qualitative *Pseudomonas aeruginosa* test. Each picture corresponds to the four reported CFU tests in the table above. After 30 min of vortexing, growth was observed in bacteria treated with BPEI AgNPs 54 ppm, AgNO₃ 20 ppm, PDADMAC 0.04 wt% solution, IAT EbNPs 0.10 wt%, IAT EbNPs 0.05 wt% coated with PDADMAC 0.02 wt%, and the supernatant of 0.05 wt% Ag-EbNPs-PDADMAC. The active agent 0.05 wt% Ag-EbNPs-PDADMAC was the only active agent resulting in no growth or 100% reduction in CFU.

Qualitative <i>P. aeruginosa</i> test										
1min vortex time or contact time	BPEI AgNPs 10ppm	BPEI AgNPs 54ppm	AgNO ₃ 10ppm	AgNO ₃ 20ppm	PDADM AC 0.02	PDADM AC 0.04	IAT0.10	IAT0.05 PDADMAC 0.02	IAT0.05 Ag+ 5ppm PDADMAC0.02	snat IAT0.05 Ag+ 5ppm PDADMAC0.02
<i>P. aeruginosa</i> growth	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
30 min vortex time or contact time	BPEI AgNPs 10ppm	BPEI AgNPs 54ppm	AgNO ₃ 10ppm	AgNO ₃ 20ppm	PDADM AC 0.02	PDADM AC 0.04	IAT0.10	IAT0.05 PDADMAC 0.02	IAT0.05 Ag+ 5ppm PDADMAC0.02	snat IAT0.05 Ag+ 5ppm PDADMAC0.02
<i>P. aeruginosa</i> growth	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes

As the control samples of PDADMAC 0.02 wt%, PDADMAC 0.04 wt%, and the IAT EbNP sample coated with PDADMAC were ineffective in terms of complete antimicrobial efficiency after 30 minutes of incubation time, the results suggest that the antimicrobial action of Ag-EbNPs-PDADMAC is delivered by silver ions. Comparing all active agents tested in terms of antimicrobial efficiency, we establish that Ag-EbNPs-PDADMAC proved most effective.

CONCLUSION

We developed a new class of nanomaterials with increased efficiency and potentially improved nanoparticle post-utilization safety. Functionalized environmentally benign nanoparticles (EbNPs) exhibit locally confined and temporarily limited bioactivity. Other than their persistent counterparts, they are predominately made from biodegradable and sustainable materials, and are synthesized via green chemistry. As these EbNPs may lose their activity due to depletion of agent, dissolution of the EbNP system, or degradation of the lignin-based matrix by the environment, they can minimize any potential nanomaterial waste hazards. In addition to the beneficial post-utilization performance, EbNPs may deliver higher efficiency in terms of active agent employed in comparison to persistent nanoparticle system. In biocidal tests on the human pathogens *E. coli* and *P. aeruginosa*, we proved that silver ion infused EbNPs with positive surface charge (Ag-EbNP-PDADMAC) exhibit significantly higher antimicrobial activities in terms of Ag equivalent than silver nanoparticles. The increased efficiency of EbNPs with functional equivalent to their persistent counterparts, may favor substitution of a wide range of applied metal nanoparticles. Moreover, the benign nature of f-EbNPs opens opportunities for new applications of nanoparticles in the agriculture, home and personal care, and pharmaceutical industry.

2 EXPERIMENTAL SECTION

Equipment.

DLS (Malvern Instruments Ltd., Nano ZS, $\lambda = 633$ nm, max. 5 mW)

Syringe pump (New Area Pump Systems, NE-4000)

UV-Vis spectrometer (Jasco UV/Vis V-550 spectrophotometer)

UV lamp (Uvitron, Sunray 400SM)

Multimeter (Mettler Toledo, S80)

Materials and chemicals used in EbNP synthesis.

Lignin. We obtained Indulin AT (IAT) powder (lot MB05) and supporting documentation from MeadWestVaco (MWV) Charleston, SC. We estimated the distribution of the main functional groups per 100 aromatic units according to the literature provided by MWV, and assigned pKa values from tables. We obtained High Purity Lignin (HPL) powder and supporting documentation from Lignol Burnaby, BC, Canada, and assigned pKa values to its functional groups accordingly. Table 7 shows the distribution of functionality of the main functional groups of both lignins.

Table 7: Main functional groups of IAT and HPL lignin, and their pKa values.

Lignin functional groups	IAT, distribution of functionality (#/100 aromatic units)	HPL, distribution of functionality (#/100 aromatic units)	pKa
Phenolic OH	68	73	10
Aliphatic OH	51	34	18
SH	9	-	10.75
COOH	16.02	-	4.2
OCH ₃	82	114	10.2
Carbonyl - O	12	-	-
Aryl alkyl ether	36	-	-
Dialkyl ether	9	-	-

Millipore water (Synergy UV); acetone (BDH, CAS# 67-64-1, lot 010612B); HNO₃ (Sigma Aldrich, CAS# 7697-37-2, lot A0294591); ethylene glycol (Sigma Aldrich, CAS# 107-21-1, grade 99+%, lot B0521395); 0.45 µm syringe filter (Thermo Scientific, nylon syringe filter 0.45 µm); magnetic stir bar (Fisher Scientific, 8-Agon stir bar 14-512-147).

HPL EbNP synthesis. The ζ-potential was measured with a Malvern disposable capillary cell DTS1061. The following measuring settings were used: the solvent was H₂O with 10% (v/v) acetone with an overall viscosity of 1.0684 cP. The effective voltage was 150 V.

IAT EbNP synthesis. The ζ-potential was measured with a Malvern disposable capillary cell DTS1061 in the size control and pH-stability studies. The following measuring settings were used: the solvent was H₂O with 10% (v/v) ethylene glycol with an overall viscosity of 1.1932 cP.

The effective voltage was 150 V. The ζ -potential was measured with a Malvern dip cell ZEN1002 in the ionic strength study. The dip cell allows ζ -potential analysis with low driving voltages. The following measuring settings were used: the solvent was H₂O. The voltage was automatically adjusted by the equipment and chosen at values below 5.0 V for all measurements.

5

IAT EbNP functionalization with Ag⁺ ions. Ag⁺ standard (Mettler Toledo, silver ISE standard 1000 ppm 51344770, lot ISEAG510L1); Ag⁺ ion selective electrode (Mettler Toledo, silver/sulfur electrode 51302822, reference filling solution C 51344752).

10 **Reference samples BPEI AgNPs and AgNO₃ solution.** Positively charged branched polyethylene imine (BPEI, Sigma Aldrich, Mw ~25000 by LS, CAS# 9002-98-6, lot MKB64206V) coated AgNPs with a z-average diameter of 20 nm were synthesized according to the literature.²⁹ The molar ratio of the final AgNP solution was chosen to be 0.5 mM BPEI: 0.5 mM AgNO₃ (Fisher Chemicals, CAS# 7761-88-8, lot 016932): 0.1 mM HEPES buffer (Sigma
15 Aldrich, CAS# 7365-45-9, lot 98H5425). 10 ml of the mixture was exposed to UV light for 120 minutes to form BPEI capped AgNPs with 54 ppm silver equivalent. The z-average diameter was determined with DLS. The pH value of the final solution was 6.3. AgNO₃ solutions were prepared from a 1000 ppm Ag⁺ reference standard. The target ppm concentrations for antimicrobial testing were reached by appropriately diluting the reference standard with
20 Millipore water.

Media used in antimicrobial testing. PBS buffer (Sigma Aldrich, CAS# 7778-77-0, lot 38H8503), LB agar (Fischer Chemicals, CAS# 9002-18-0), LB broth (Acros 61187-5000, lot B012260G).

25

Example 2

30 One of the most widely used classes of nanomaterials today is the silver nanoparticles (AgNPs), which exhibit general antimicrobial, antispore and antifungal activity, while being of low toxicity to humans. The application of Ag nanoparticles, however, has met a number of serious problems, due to their relatively large cost and the rapidly growing concerns about the environmental and human dangers by the persistent nanoparticles released post application. We
35 have pioneered a set of new ideas that resulted in the demonstration of a novel class of

functionalized, environmentally-benign, nanoparticles (EbNPs) as highly efficient microbicidal substitutes of the AgNPs (Figure 20). These particles are made of biodegradable and environmentally benign biopolymers such as lignin, and are infused with an optimal amount of silver in the form of adsorbed Ag^+ ions. The active Ag^+ ions are released only during the targeted
5 adsorption of the polyelectrolyte-coated particles onto bacterial targets. We have shown that the bactericidal action of these silver-loaded, surface functionalized particles exceeds the one of the common Ag nanoparticles and that the new EbNPs are capable of killing a broad spectrum of microbes. A more detailed description of these preliminary results is provided in the main section of this document. The nanoparticles will become depleted of Ag^+ ions shortly after use
10 rendering the nanosystem benign. If the EbNPs are released in the environment concerns related to nanowaste toxicity are minimized as the particles will degrade, for they are made from natural biopolymers. Both the nanoparticles and the processes for their fabrication are simple and inexpensive. This approach is not limited only to bactericidal silver ions as active agent, but can be applied to the development of many new biopolymer particles with antiviral, antifungal,
15 antitoxin, anticancer, chemical decontamination and other functionalities.

The environmentally benign nanoparticles (EbNPs) that we have developed address these safety concerns associated with nanosystems without sacrificing the powerful nano-scale functionality. The synthesis of pH-stable IAT-based environmentally biodegradable nanoparticles in ethylene glycol may be accomplished by a process that is simple, predominantly
20 water-based, and does not include harsh organic solvents or chemical agents (Frangville, C.; Rutkevičius, M.; Richter, A. P.; Velez, O. D.; Stoyanov, S. D.; Paunov, V. N., Fabrication of Environmentally Biodegradable Lignin Nanoparticles. *ChemPhysChem* **2012**, *13* (18), 4235-4243).

We further proved that by infusing IAT EbNPs with functional metal ions, such as
25 antimicrobial silver ions, and additional surface modification, such as switching the surface charge from negative to positive, it is possible to synthesize degradable nanoparticles that match the nanoparticle functionality of their respective PNPs, while increasing utilization and post-utilization safety. First, we synthesize negatively charged IAT EbNPs suitable for functionalization with cationic metal ions. In the next step, we adsorb antimicrobial silver ions
30 from water solution of silver nitrate. Finally, we reverse the surface potential of the particles from negative to positive via adsorption of a positively charged polyelectrolyte. We use a relatively benign multifunctional cationic polyelectrolyte, polydiallyldimethylammonium chloride [PDADMAC], which has been frequently employed in environmental applications such as water treatment, in consumer products such as cosmetics, and in biological application

including insecticides and algacides (Wandrey et al., Diallyldimethylammonium Chloride and its Polymers. In *Radical Polymerisation Polyelectrolytes*, Capek et al., Eds. Springer Berlin Heidelberg: 1999; Vol. 145, pp 123-183). Besides the surface charge modification, the PDADMAC layer may protect the particle system from unintended Ag^+ ion depletion. In addition, as quarternized amines are known to exhibit antimicrobial effects, thus PDADMAC may potentially increase the antimicrobial efficiency of the EbNPs (Zhao, X.; Zhang, Y., - Bacteria-removing and Bactericidal Efficiencies of PDADMAC Composite Coagulants in Enhanced Coagulation Treatment. - *CLEAN – Soil, Air, Water* 2012). Hence, the antimicrobial EbNPs (Ag-EbNPs-PDADMAC) with functional equivalency to AgNPs consist of (1) a biodegradable EbNP core, (2) highly antimicrobial silver ions as active agent, and (3) a surface charge modifier. At contact with the cells, the particles can release antimicrobial silver ions, which can be transfer into the cell, to perform the desired antimicrobial function leading to bacteria cell death. In contrast to the metallic silver in AgNPs, silver in Ag-EbNPs-PDADMAC is already available in its ionic form and therefore, may be transferred to the cell more readily. This process may result in rapid silver ion depletion of the Ag-EbNPs-PDADMAC system. At the end of the lifecycle, the Ag-EbNPs-PDADMAC system, which is depleted of silver ions, is rendered inactive and will degrade over time.

Antimicrobial testing and comparison of AgNPs with silver-infused EbNPs. We compared the antimicrobial activity of Ag-EbNPs-PDADMAC with the one of positively charged branched polyethylene imine AgNPs (BPEI AgNPs) and AgNO_3 solutions. We performed quantitative antimicrobial tests on Gram-negative *E. coli* BL21 (DE3), a common human pathogen, and qualitative tests on Gram-negative *P. aeruginosa*, a human pathogen not susceptible to antimicrobial amines such as BPEI and PDADMAC. Therefore, any antimicrobial activity in the *P. aeruginosa* tests will predominantly stem from silver. The testing procedure are reported in Example 1.

The quantitative antimicrobial efficiencies of each active agent in the *E. coli* tests are compared in Figure 21. The microbicidal efficiency of six different samples with increasing Ag ppm equivalent ranging from 0 ppm to 54 ppm was investigated. The graphs show the reduction efficiency at two time points, 1 minute and 30 minutes. The weight percentages of the control samples and the silver contents in the active agents were chosen to show antimicrobial thresholds and to facilitate comparisons between the samples. Native IAT EbNPs without Ag^+ functionalization and surface modification did not result in any observable reductions in CFU (not reported), which suggests that the native IAT EbNPs are benign. Also, IAT EbNPs loaded with Ag^+ but without PDADMAC coating did not result in significant reduction of CFU after 1

minute (0%) and 30 minutes (5%). We believe that the low antimicrobial efficiency may be contributed to the negative surface charge of these EbNPs, which may hinder them from overcoming the electrostatic barrier between the particles and the bacteria. IAT EbNPs coated with PDADMAC resulted in strong reduction of CFU after an exposure time of 30 minutes, which may be attributed to the antimicrobial effect of the quarterly amine PDADMAC. The Ag⁺-loaded and surface-functionalized sample, Ag-EbNPs-PDADMAC, exhibited strong reduction in CFU, prevalent after 1 minute exposure time. The corresponding supernatant of Ag-EbNPs-PDADMAC exhibited no observable effect after 1 minute. BPEI AgNPs and AgNO₃ solutions exhibited antimicrobial effects at 20 ppm Ag and 40 ppm Ag respectively.

Overall, the Ag-EbNPs-PDADMAC sample outperformed the BPEI AgNPs and AgNO₃ samples in terms of antimicrobial efficiency normalized on Ag ppm equivalent. Table 8 presents a comparison of the properties of common AgNPs with our novel silver-infused EbNPs.

Table 1: Comparison of conventional AgNPs with the new silver-infused EbNPs.

Parameter	Silver Nanoparticles (AgNPs)	New functionalized EbNPs
Broad spectrum antimicrobial/antispore/antiviral agent	Yes	Yes
Efficiency	++	+++ (up to 10X higher efficiency)
Silver-content (priority pollutant)	High	Low (reduction factor up to 10)
Matrix	Persistent (metallic Ag based)	Degradable (biopolymer-based)
Suitability for customized functionality	Low (one function)	High (functionalization with other active agents possible)
Regulatory Concerns	Yes (under scrutiny by US EPA)	Minimized (collaboration with EPA)
Comparative activity towards mammalian cells	Medium (tested at US EPA)	Low (tested at US EPA)
Bioactivity post-utilization	Yes (metallic core remains active)	Minimized (depleted of active agent)
Scalability	Difficult (mostly batch production)	Yes (continuous flow production possible)
Comparative concentration [wt%] in solution	Low	High (10 to 100 higher)
Price	\$400/g AgNPs in solution (estimate, PlasmaChem GmbH, 2012)	\$4.0/g EbNPs in solution (conservative estimate)

As mentioned above, the qualitative antimicrobial test on *P. aeruginosa* can distinguish the antimicrobial effect of PDADMAC from the effect of silver (Figure 22). BPEI AgNPs and AgNO₃ solutions exhibited no complete antimicrobial effect after 30 minutes incubation time. The control sample IAT EbNP and the IAT EbNPs coated with PDADMAC also did not result in any significant effect. Thus, the sample Ag-EbNPs-PDADMAC was the only one that exhibited complete or 100% antimicrobial efficiency after 30 minutes. The supernatant of the sample Ag-

EbNPs-PDADMAC did not show any antimicrobial effect after 30 minutes of incubation time. The results suggest that the antimicrobial action of Ag-EbNPs-PDADMAC is delivered by silver ions. Comparing all active agents tested in terms of antimicrobial efficiency, we establish that Ag-EbNPs-PDADMAC again proved most effective.

Hypothesis of the antibacterial mechanism of Ag-EbNPs-PDADMAC. The antibacterial effect of Ag-EbNPs-PDADMAC, without wishing to be bound by theory, is believed to be a combinatorial effect of the antimicrobial properties of Ag^+ ions and of the quarternized amine PDADMAC. For the bacteria not susceptible for bactericidal amines such as *P. aeruginosa* (Adair et al., Resistance of Pseudomonas to Quaternary Ammonium Compounds. I. Growth in Benzalkonium Chloride Solution. *Applied Microbiology* 1969, 18 (3), 299-302; Langsrud et al., Intrinsic and acquired resistance to quaternary ammonium compounds in food-related Pseudomonas spp. *Journal of Applied Microbiology* 2003, 95 (4), 874-882), the antimicrobial effect is based on the bactericidal activity of silver-ions. We suggest that the Ag^+ ions are weakly bound to the EbNP binding sites and locally concentrated on the EbNP surface. These Ag^+ ions may be surface active and could be released upon contact with a bacteria cell membrane. A possible mechanism of the antimicrobial Ag-EbNPs-PDADMAC activity is illustrated in Figure 23. Ag-EbNPs-PDADMAC particles are electrostatically attracted to the negatively charged bacteria cell membrane, and will eventually adhere to it. As the control sample of EbNPs with PDADMAC but without Ag^+ ions did not show antimicrobial effects towards *P. aeruginosa*, we suggest that the particles by themselves may not destroy the integrity of the cell membrane, which could result in cell lyses and therefore cell death. Hence, we suggest that the antimicrobial effect stems predominately from Ag^+ ions, which may be released by the EbNP system towards the bacteria. As the Ag^+ ions may eventually migrate into the cell, they could adversely affect bacteria cell functions and therefore, lead to cell death.

Conclusions. We developed a new class of microbicidal nanoparticles with increased efficiency and improved post-utilization safety. In contrast to AgNPs, the Ag-EbNPs-PDADMAC system is synthesized via green chemistry and employs degradable, benign and sustainable materials. Since these EbNPs can promote significantly higher antimicrobial activities in terms of Ag equivalents in comparison to persistent AgNPs, their environmental footprint is largely reduced. Furthermore, antimicrobial EbNPs are benign towards mammalian cells in comparison to AgNPs at equivalent silver concentration. As the EbNP technology is

flexible and may be applied to a wide range of active agents, functionalized EbNPs may be suitable to substitute a wide range of applied metal nanoparticles.

Additional data, examples and embodiments may be found in **Appendix A** attached to the specification of U.S. provisional patent application no. 61/776,274, filed March 11, 2014, the benefit of which application is claimed by the present application, and the contents of which application are incorporated by reference herein in its entirety.

It is to be understood that, while the invention has been described in conjunction with the detailed description, thereof, the foregoing description is intended to illustrate and not limit the scope of the invention. Other aspects, advantages, and modifications of the invention are within the scope of the claims set forth below. All publications, patents, and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

What is claimed is:

1. A nanoparticle comprising:
a biodegradable biopolymer core;
an antiviral or cytotoxic agent loaded on the biodegradable biopolymer core; and
a bioadhesive layer coating the biodegradable biopolymer core and the antiviral or cytotoxic agent loaded thereon.
2. The nanoparticle of claim 1, wherein the biodegradable biopolymer core is a lignin or a modified lignin.
3. The nanoparticle of claim 1, wherein the biodegradable biopolymer core is a plant- or animal-derived biopolymer.
4. The nanoparticle of claim 3, wherein the plant- or animal-derived biopolymer is a cellulose, a chitin, a chitosan, a hemicellulose, a lignocellulose, a modified cellulose, a modified chitosan, a modified lignin, a protein, or a combination thereof.
5. The nanoparticle of claim 4, wherein the chitosan is a medium or high molecular weight chitosan or a derivative thereof.
6. The nanoparticle of claim 4, wherein the modified cellulose is cellulose acetate, cellulose nitrate, cellulose propionate, ethyl cellulose, hydroxyethyl cellulose, hydroxypropyl methylcellulose phthalate, hydroxypropyl methylcellulose acetate succinate, or methyl cellulose, or a derivative thereof.
7. The nanoparticle of claim 4, wherein the modified chitosan is a low molecular weight chitosan, a chitosan with amino groups in the backbone, or a derivative thereof.
8. The nanoparticle of claim 4, wherein the modified lignin is sulfonated (Indulin C) or unsulfonated (Indulin AT, High Purity Lignin) lignin.
9. The nanoparticle of claim 1, wherein the antiviral or cytotoxic agent is an antiviral agent.
10. The nanoparticle of claim 1, wherein the antiviral or cytotoxic agent is a biocide, a cationic metal, a catalyst, a fumigant, a herbicide, a pesticide, a photocatalyst, or a semiconductor.
11. The nanoparticle of claim 10, wherein the biocide is an algacide, a bactericide bactericidal/permeability-increasing protein (BPI), or a fungicide.
12. The nanoparticle of claim 11, wherein the fungicide is captan, chlorothalonil, cyrodinil, folpet, mepanipyrim, pyrimethanil, sulfur, or vinclozolin.
13. The nanoparticle of claim 11, wherein the fungicide is an ethylenebisdithiocarbamate or a natural fungicide.

14. The nanoparticle of claim 13, wherein the ethylenebisdithiocarbamate is mancozeb, maneb, metiram, nabam, or zineb.
15. The nanoparticle of claim 13, wherein the natural fungicide is *ampelomuces quisqualis*, cinnamaldehyde, cinnamon essential oil, jojoba oil, monocerin, neem oil, rosemary oil, or tee tree oil.
16. The nanoparticle of claim 10, wherein the cationic metal is Ag^+ , Ag^{2+} , Ag^{3+} , Co^{2+} , Cu^{2+} , Fe^{2+} , Ni^{2+} , or Zn^{2+} .
17. The nanoparticle of claim 10, wherein the fumigant is 1,3-dichloropropene, chloropicrin, formaldehyde, iodoform, metam sodium, methyl bromide, methyl iodide, methyl isocyanate, phosphine, or sulfuryl fluoride.
18. The nanoparticle of claim 10, wherein the herbicide is glyphosate, triclopyr, 1,1'-dimethyl-4,4'-bipyridinium ion (paraquat) or a chemical derivative, analogue or salt thereof.
19. The nanoparticle of claim 10, wherein the herbicide is a chloracetanilide herbicide, glyphosate herbicide, an imidazolinone herbicide, an organic herbicide, a phenoxy herbicide, a phenylurea herbicide, a triazine herbicide, or a triazolopyrimidine herbicide.
20. The nanoparticle of claim 19, wherein the chloracetanilide herbicide is acetochlor, alachlor, butachlor, metolachlor, or propachlor.
21. The nanoparticle of claim 19, wherein the imidazolinone herbicide is imazapyr, imazamethabenz-methyl, imazapic, imazethapyr, imazamox or imazaquin.
22. The nanoparticle of claim 19, wherein the organic herbicide is corn gluten meal, vinegar, D-limonene, or monocerin.
23. The nanoparticle of claim 19, wherein the phenoxy herbicide is 2,4-Dichlorophenoxyacetic acid, 2,4,5-Trichlorophenoxyacetic acid, 2-Methyl-4-chlorophenoxyacetic acid, 2-(2-Methyl-4-chlorophenoxy)propionic acids, 2-(2,4-Dichlorophenoxy)propionic acid, or 2,4-Dichlorophenoxy)butyric acid.
24. The nanoparticle of claim 19, wherein the phenylurea herbicide is *N*'-(3,4-dichlorophenyl)-*N*, *N*-dimethylurea (diuron), 1,1-dimethyl-3-[3-(trifluoromethyl)phenyl] (fluometuron), or *N,N*-dimethyl-*N*'-[4-(1-methylethyl)phenyl] (isoproturon).
25. The nanoparticle of claim 19, wherein the triazine herbicide is ametryn, atrazine, cyanazine, prometon, prometryn, propazine, simazine, terbuthylazine, or terbutryn.
26. The nanoparticle of claim 19, wherein the triazolopyrimidine herbicide is clorasulam-methyl, diclosulam, florasulam, flumetsulam, metosulam, penoxsulama, or pyroxsulama.
27. The nanoparticle of claim 10, wherein the pesticide is an avicide, an insecticide, a miticide, a molluscicide, a nematocide, or a rodenticide.

28. The nanoparticle of claim 27, wherein the insecticide is a carbamate or a pyrethroid insecticide.
29. The nanoparticle of claim 28, wherein the carbamate insecticide is aldicarb, carbaryl, carbofuran, formetanate, methiocarb, oxamyl, pirimicarb, propoxur, or thiodicarb.
30. The nanoparticle of claim 28, wherein the pyrethroid insecticide is allethrin, bifenthrin, cyhalothrin, lambda-cyhalothrin, cypermethrin, cyfluthrin, deltamethrin, etofenprox, fenvalerate, permethrin, phenothrin, prallethrin, pesmethrin, tetramethrin, tralomethrin, or transfluthrin.
31. The nanoparticle of claim 27, wherein the rodenticide is an anticoagulants, brodifacouma, bromadiolonea, chlorophacinone, difethialone, diphacinone, pindone, warfarin, nonanticoagulant, bromethalin, cholecalciferol, strychnine, or zinc phosphide
32. The nanoparticle of claim 10, wherein the semiconductor is Ag_2S , CdS , CdSe , CdTe , Cu_2S , CuCl , CuO , Fe_2O_3 , Fe_2S , Fe_3O_4 (α , β , γ , ϵ), FeO , NiO , TiO_2 , ZnO , ZnS , ZnSe , or ZnTe .
33. The nanoparticle of claim 1, wherein the bioadhesive layer is a cationic polymer.
34. The nanoparticle of claim 33, wherein the cationic polymer is a polyamino-polymer.
35. The nanoparticle of claim 34, wherein the polyamino-polymer is branched polyethyleneimine (BPEI), polyallylamine hydrochloride (PAH), polydiallyldimethylammonium chloride (PDADMAC), polyethoxylated tallow amine (POEA), polyethyleneimine (PEI), or polylysine.
36. The nanoparticle of claim 1, wherein the bioadhesive layer comprises primary, secondary, tertiary, or quaternized amines (benzalkonium chloride, benzethonium chloride).
37. The nanoparticle in claim 1, where the bioadhesive layer comprises carbohydrates, polypeptides, lectins, proteins, or antibodies or other molecules or materials with affinity to microbes, viruses or seeds.
38. The nanoparticle in claim 1, where the bioadhesive layer comprises nanohairs or nanolatches.
39. The nanoparticle of claim 1, wherein the nanoparticle has a diameter of about 10 nm to about 500 nm.
40. The nanoparticle of claim 39, wherein the nanoparticle has a diameter of about 20 nm to about 100 nm.
41. The nanoparticle of claim 40, wherein the nanoparticle has a diameter of about 50 nm to about 80 nm.
42. A coated article comprising a surface wherein at least a portion of the surface is coated with the nanoparticle of claim 1.

43. The coated article of claim 42, wherein the coated article is an air filter, an article of clothing, an article of hygiene, a building material, a face mask, a food stuff package, a medical device, or a seed.
44. The coated article of claim 43, wherein the medical device is bandage, a biological implant, a dressing, a medical scaffold, a surgical instrument, or a wound covering.
45. The use of the nanoparticle of claim 1 to impart antiviral or cytotoxic properties to a substrate.
46. A method for fabricating a nanoparticle, the method comprising:
contacting a solvent containing a dissolved biodegradable biopolymer with an anti-solvent so as to form a biodegradable biopolymer core;
loading an antiviral or cytotoxic agent on the biodegradable biopolymer core; and
coating the biodegradable biopolymer core and the antiviral or cytotoxic agent with a bioadhesive layer.
47. A method for fabricating a nanoparticle, the method comprising:
altering the pH of a suitable solvent containing a dissolved biodegradable biopolymer so as to form a biodegradable biopolymer core;
loading an antiviral or cytotoxic agent on the biodegradable biopolymer core; and
coating the biodegradable biopolymer core and the antiviral or cytotoxic agent with a bioadhesive layer.
48. A method for fabricating a nanoparticle, the method comprising:
contacting an organic solvent containing a dissolved biodegradable biopolymer with an aqueous solvent under suitable pH conditions so as to form a biodegradable biopolymer core;
loading an antiviral or cytotoxic agent on the biodegradable biopolymer core; and
coating the biodegradable biopolymer core and the antiviral or cytotoxic agent with a bioadhesive layer.
49. A method for fabricating a nanoparticle, the method comprising:
contacting an aqueous containing a dissolved biodegradable biopolymer with a polyelectrolyte under suitable conditions so as to form a biodegradable biopolymer core;
loading an antiviral or cytotoxic agent on the biodegradable biopolymer core; and
coating the biodegradable biopolymer core and the antiviral or cytotoxic agent with a bioadhesive layer.
50. A nanoparticle fabricated according to the method of claim 46.
51. A nanoparticle fabricated according to the method of claim 47.
52. A nanoparticle fabricated according to the method of claim 48.

53. A nanoparticle fabricated according to the method of claim 49.
54. The nanoparticle of claim 4, wherein the protein is a prolamin or a gluten.
55. The nanoparticle of claim 54, wherein the prolamin is gliadin, hordein, secalin, zein, kafirin or avenin.
56. The nanoparticle of claim 54, wherein the gluten is gliadin or glutenin.
57. The nanoparticle of claim 1, wherein the biodegradable biopolymer core is a byproduct of lignin degradation.
58. The nanoparticle of claim 57, wherein the byproduct of lignin degradation is humic acid.
59. The nanoparticle of claim 1, wherein the biodegradable biopolymer core is a synthetic polyphenol, a natural phenolic polymer, or a linear, branched, or cross-linked polysaccharide, or a derivative thereof.
60. The nanoparticle of claim 1, wherein the biodegradable biopolymer core is a linear, branched, or cross-linked biopolymer of natural or technical origin (i.e. physically or chemically modified during processing of natural compounds and appearing usually as bi-product or waste stream of such process).
61. The nanoparticle of claim 1, wherein the biodegradable biopolymer core is a large molecular weight polyphenols or natural phenolic polymers like lignin or modified lignin or a combination thereof.
62. The method of fabrication of claim 48, wherein the organic solvent is acetone, ethylene glycol, propylene glycol, glycerol, methyl acetate, ethyl acetate, methanol, ethanol, hexanol, petrol ether, toluene, benzene, turpentine, dichlormethane, chloroform, formic acid, isopropanol, polyethylene oxide, or a combination thereof.
63. The nanoparticle of claim 11, wherein the bactericide is an anionic antimicrobial peptide or an anionic antimicrobial protein (AAMPs).
64. The nanoparticle of claim 11, wherein the bactericide is an antibacterial peptide (gramicidin, magainins, melittin, defensins, amphotericins or nystatin).
65. The nanoparticle of claim 1, wherein the bioadhesive layer is a silicone based surfactant.
66. The nanoparticle of claim 65, wherein the silicone based surfactant is silicone blends with polysiloxane chains
67. The nanoparticle of claim 1, wherein the bioadhesive layer is a oil based surfactant.
68. The nanoparticle of claim 67, wherein the oil based surfactant is a vegetable oil, methylated vegetable oil, seed oils, crop oils, petroleum based oils, silicon based oils, and blends of these.

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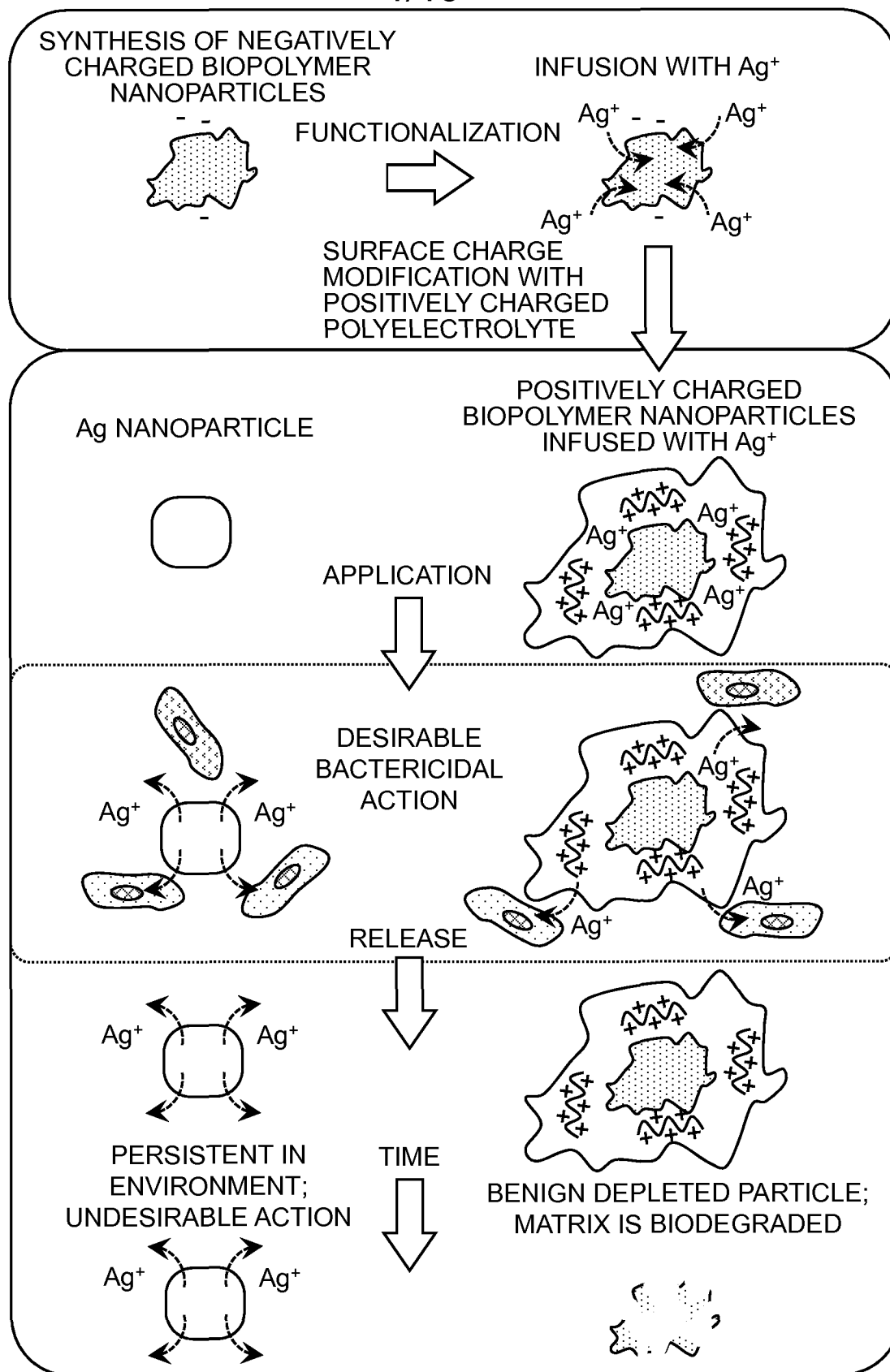


Figure 1

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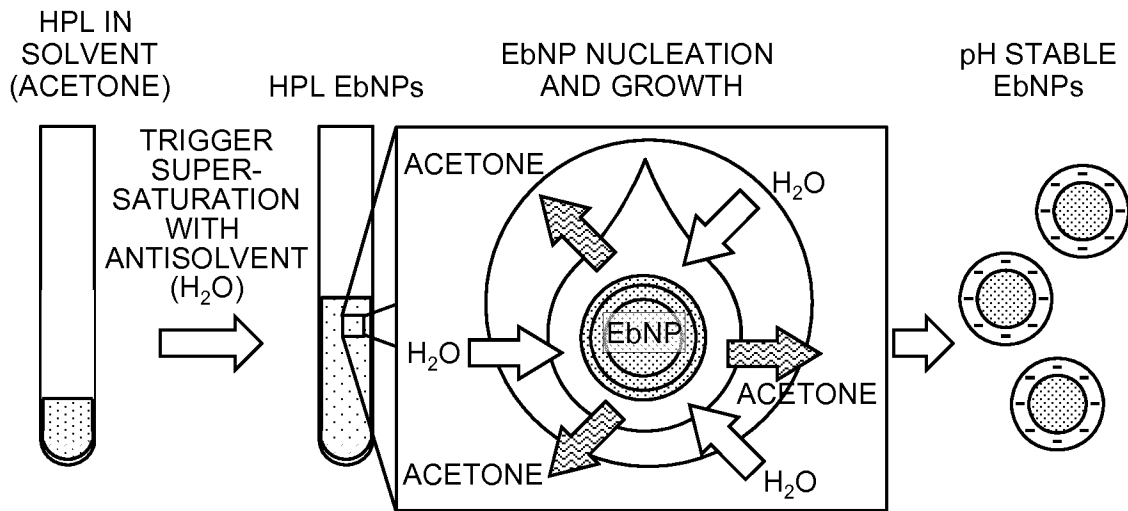


Figure 2

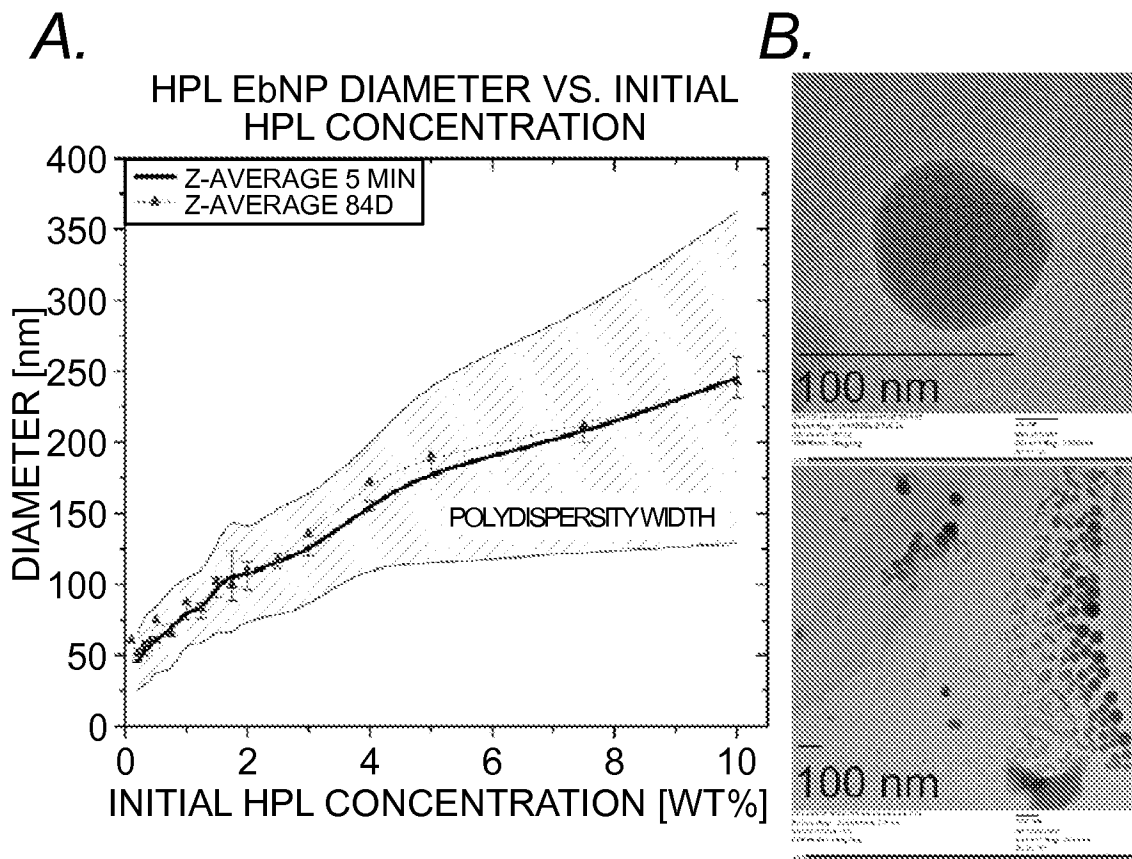


Figure 3

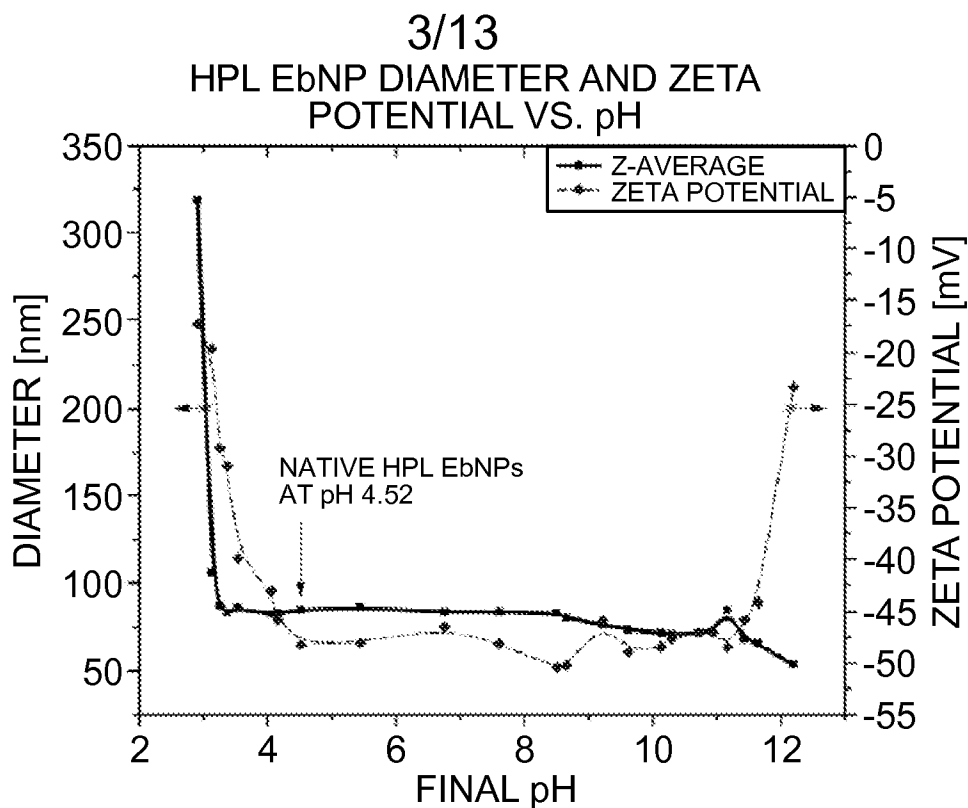
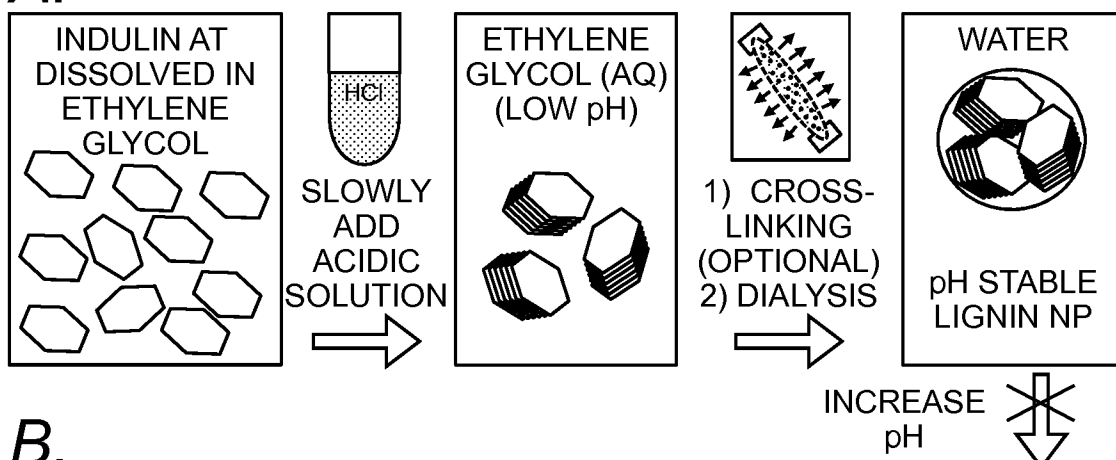


Figure 4

A.



B.

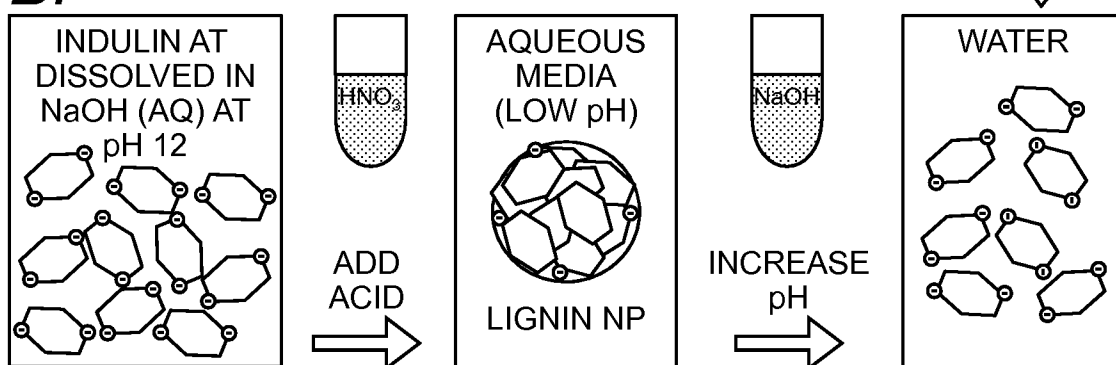


Figure 5

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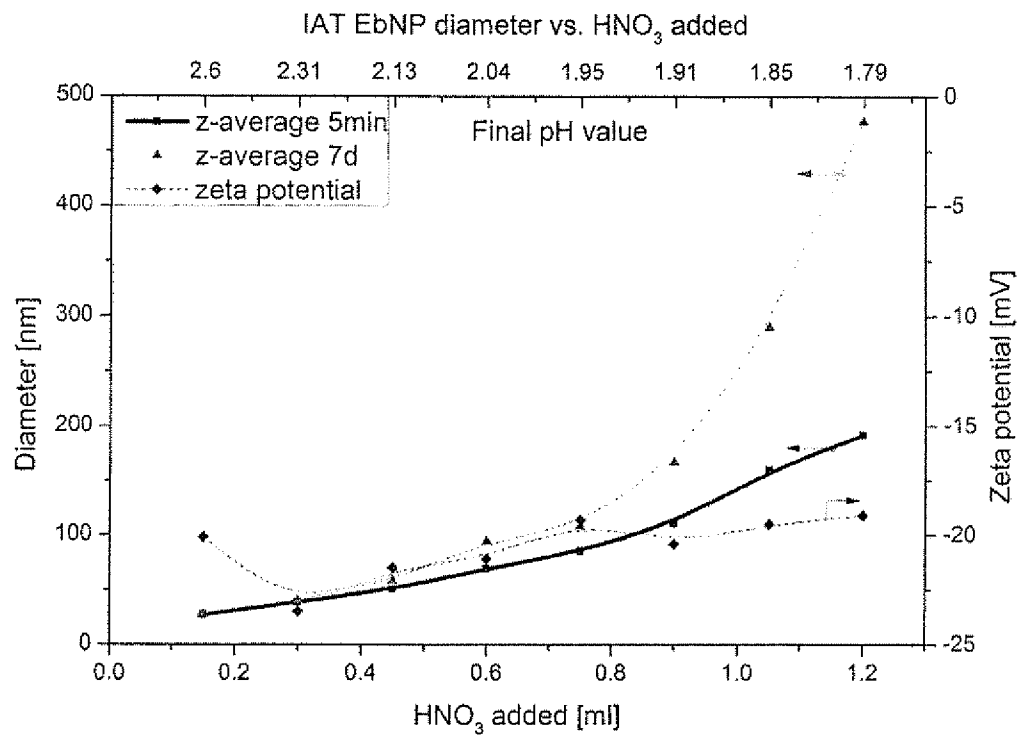


Figure 6

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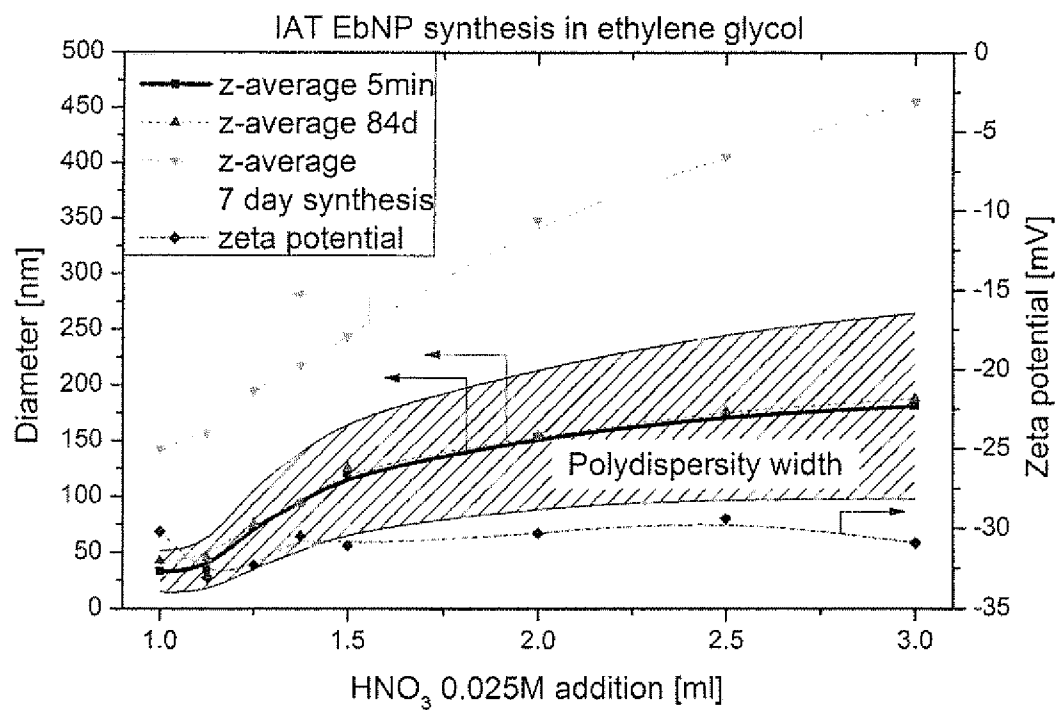


Figure 7

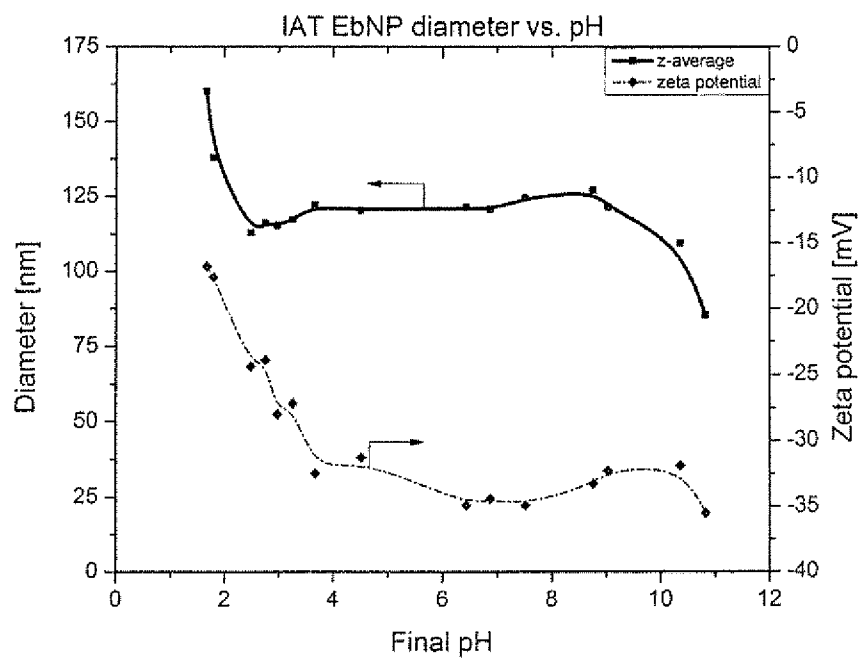


Figure 8

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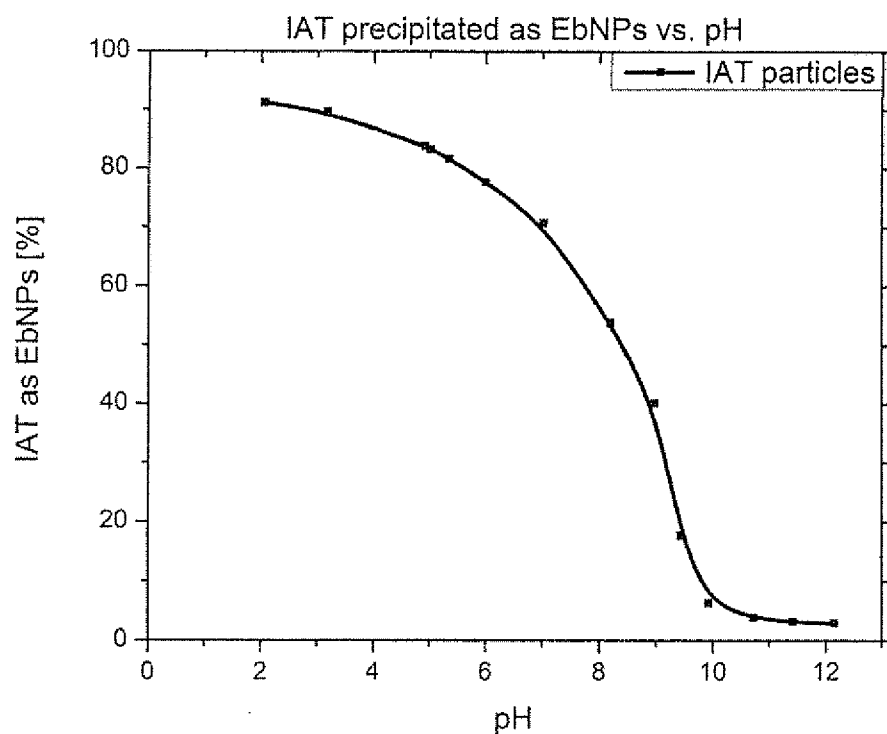


Figure 9

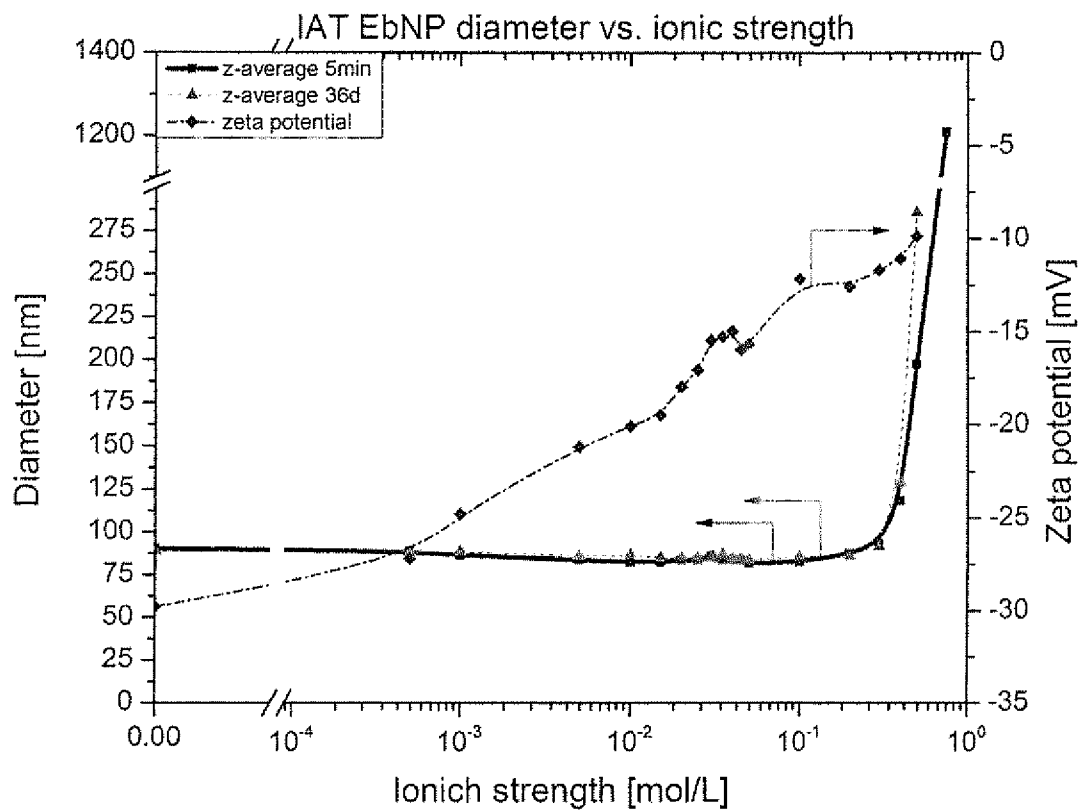


Figure 10

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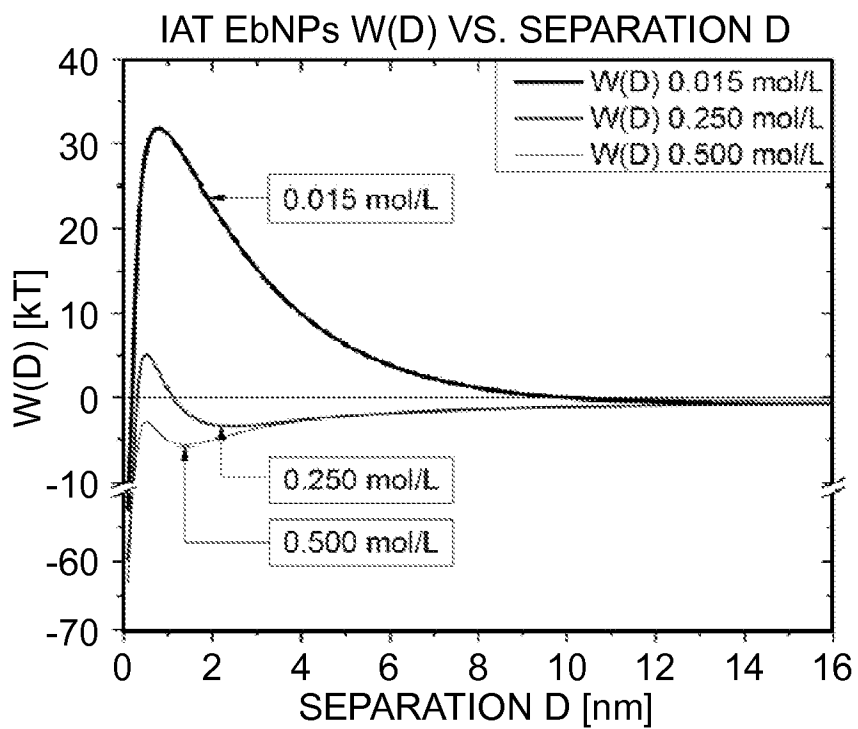


Figure 11

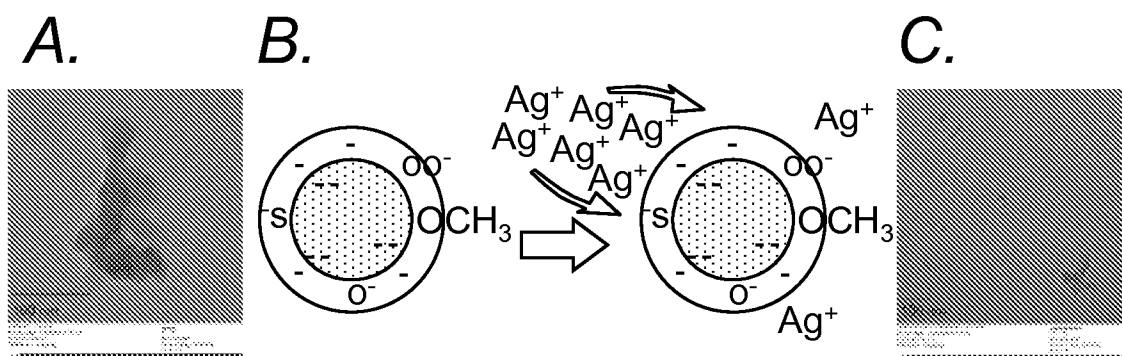


Figure 12

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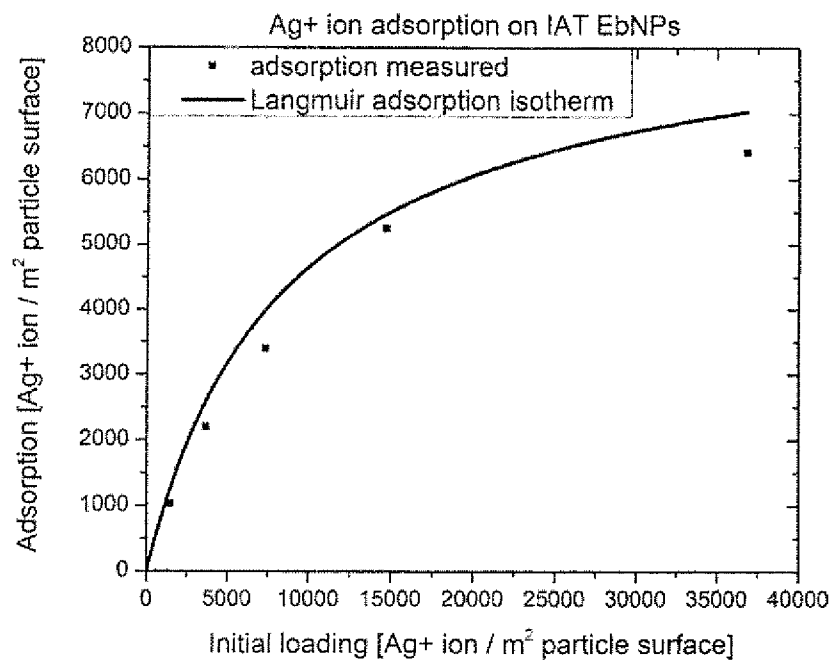


Figure 13

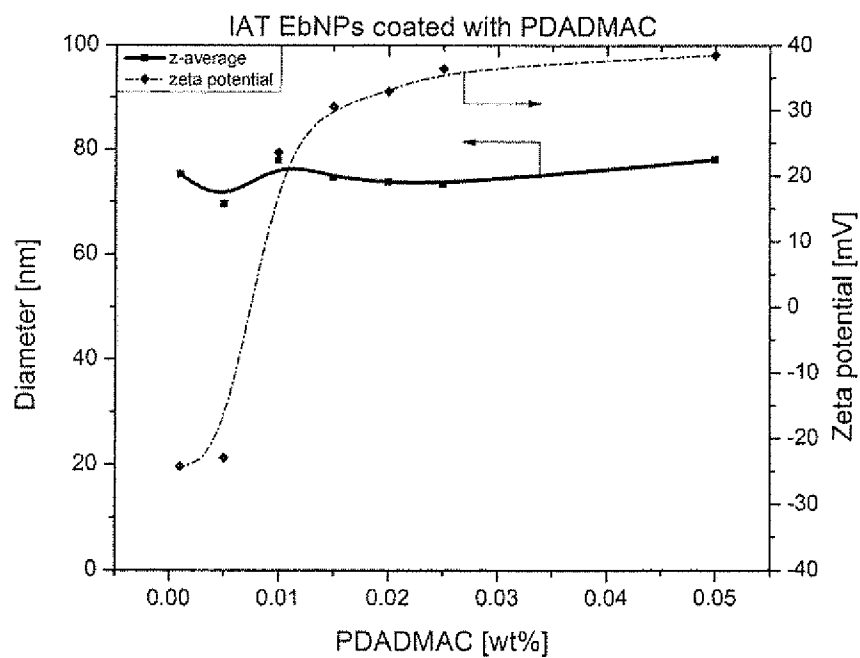


Figure 14

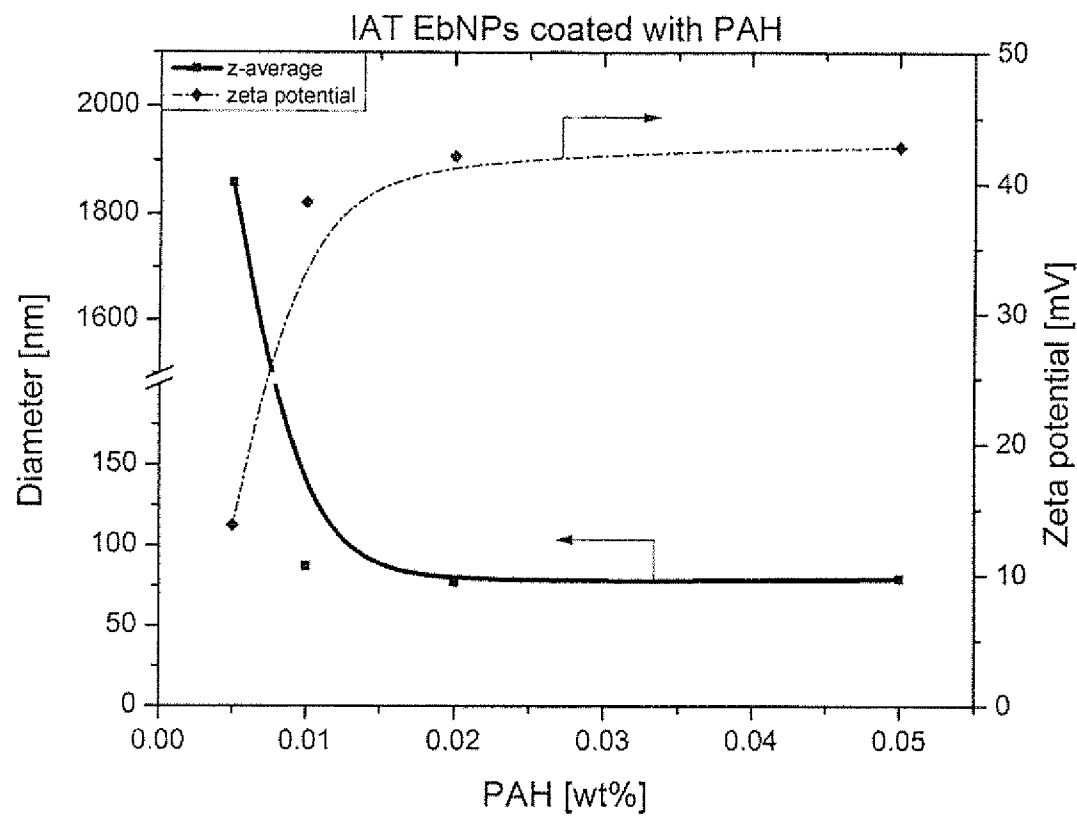


Figure 15

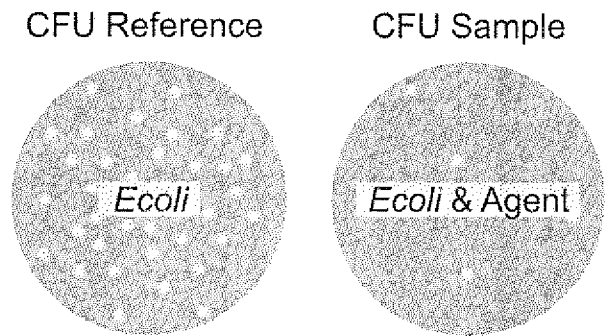
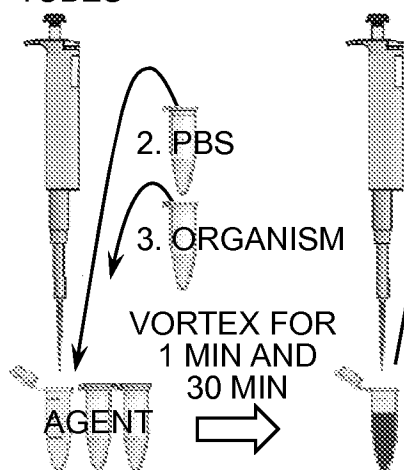


Figure 16

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WET METHOD

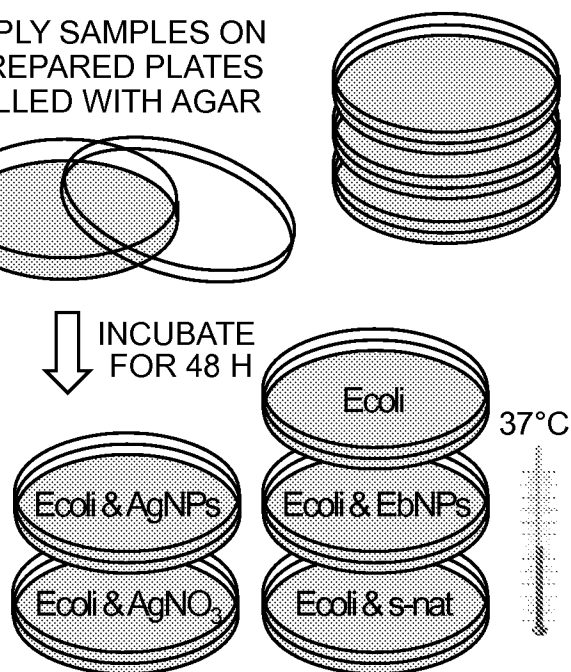
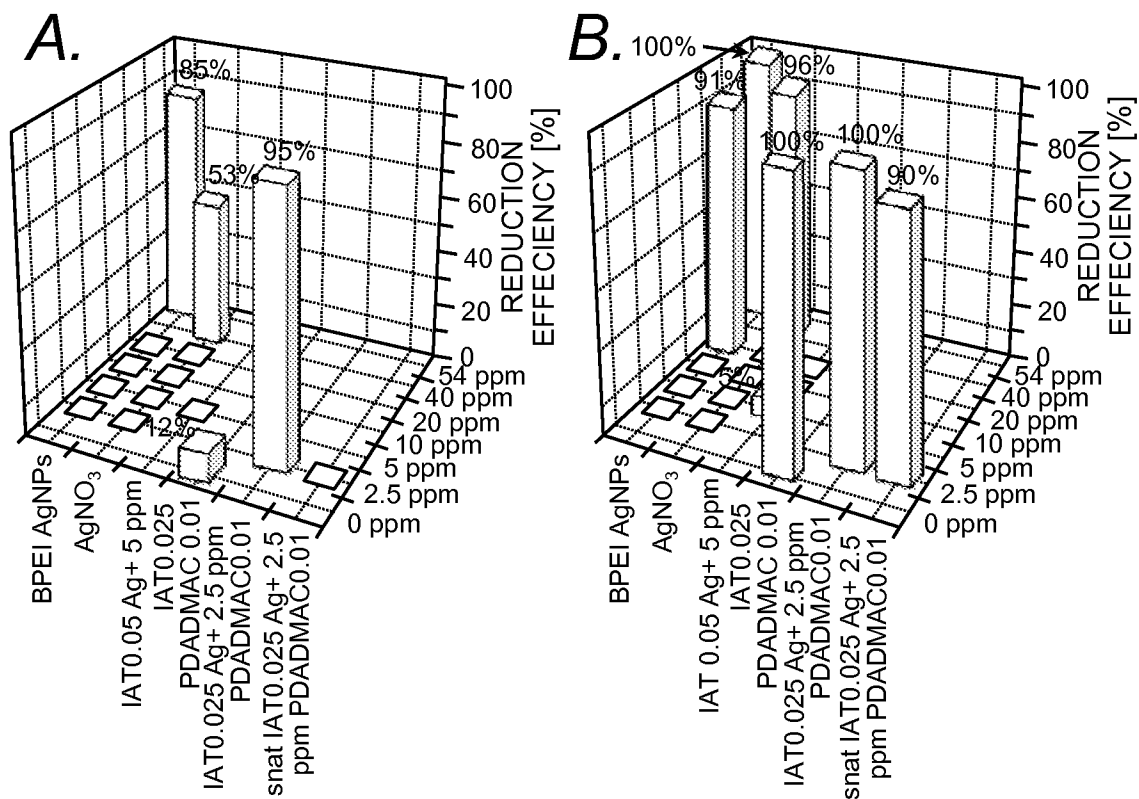
1. PIPETTE ACTIVE AGENT (EbNPs, EbNP S-nat, AgNPs, AgNO₃) INTO CENTRIFUGAL TUBES



EbNP, Snat, EbNPs, AgNPs, etc.

APPLY SAMPLES ON PREPARED PLATES FILLED WITH AGAR

INCUBATE FOR 48 H

**Figure 17****Figure 18**

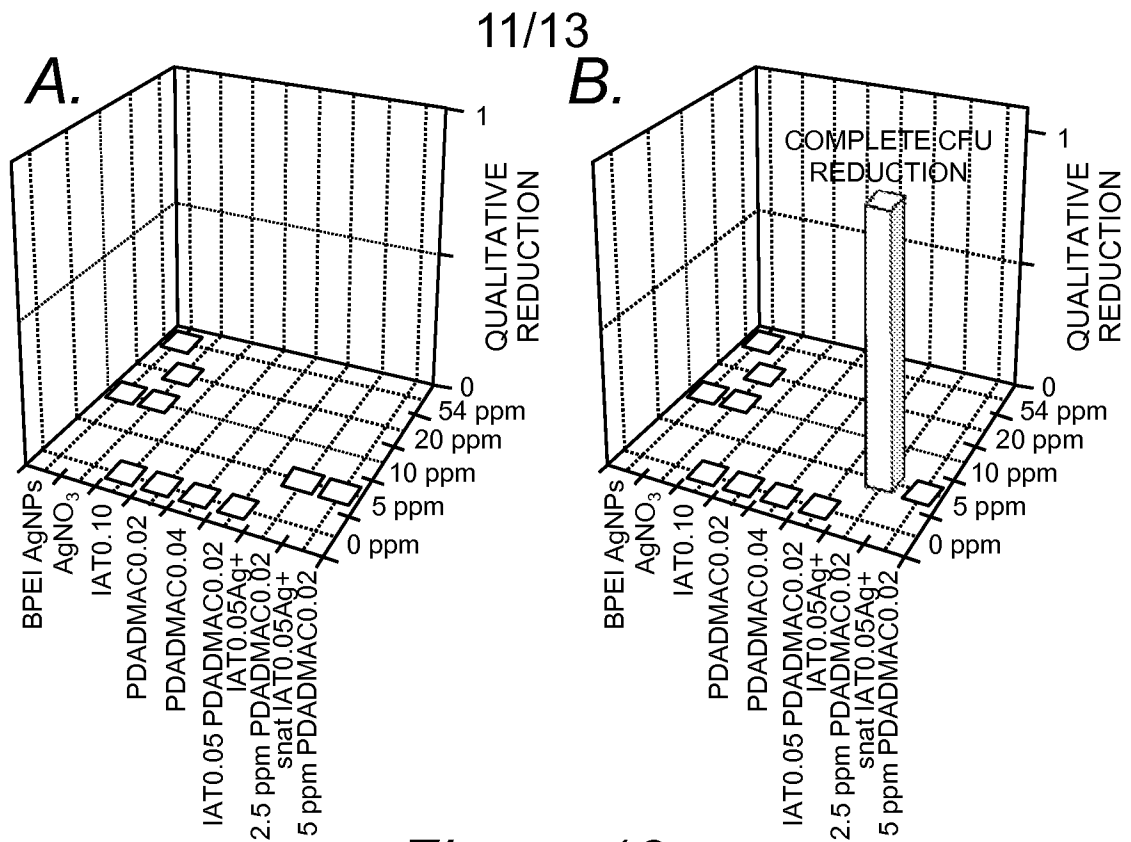


Figure 19

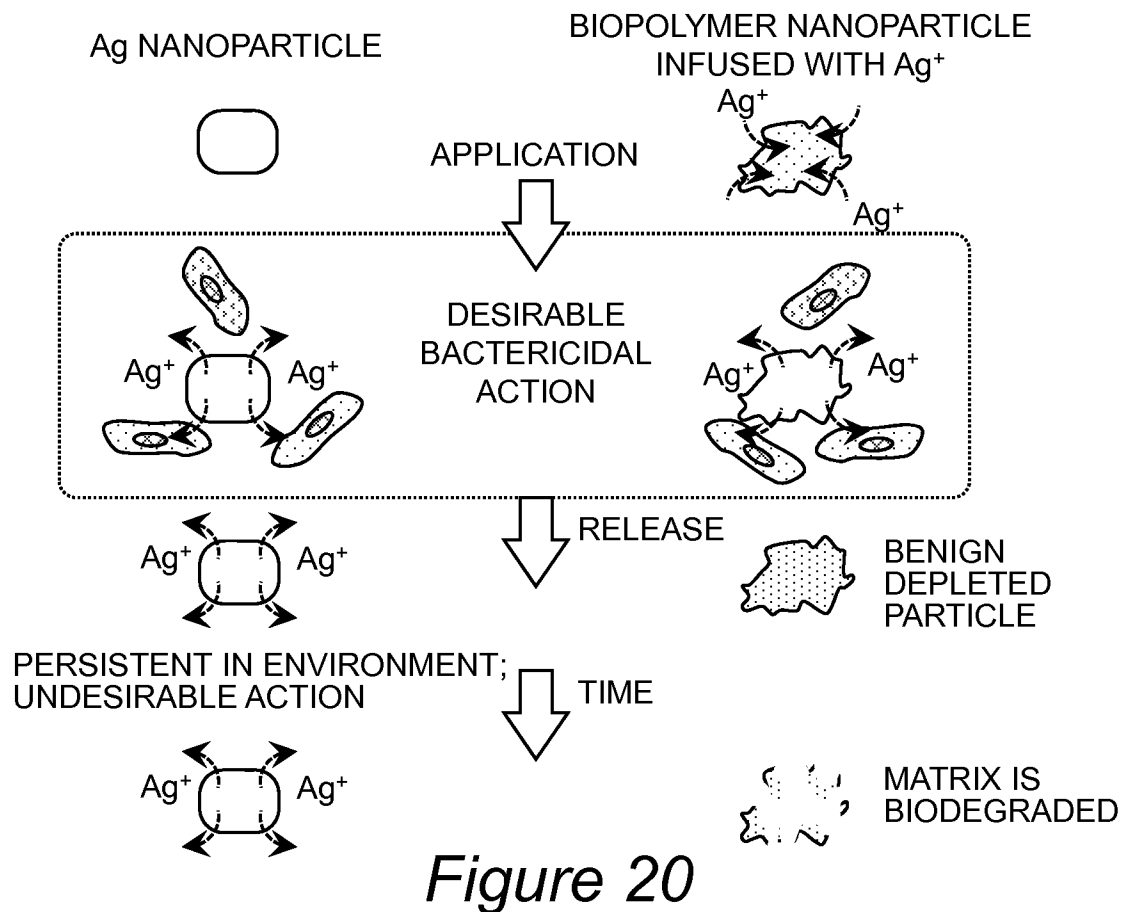


Figure 20

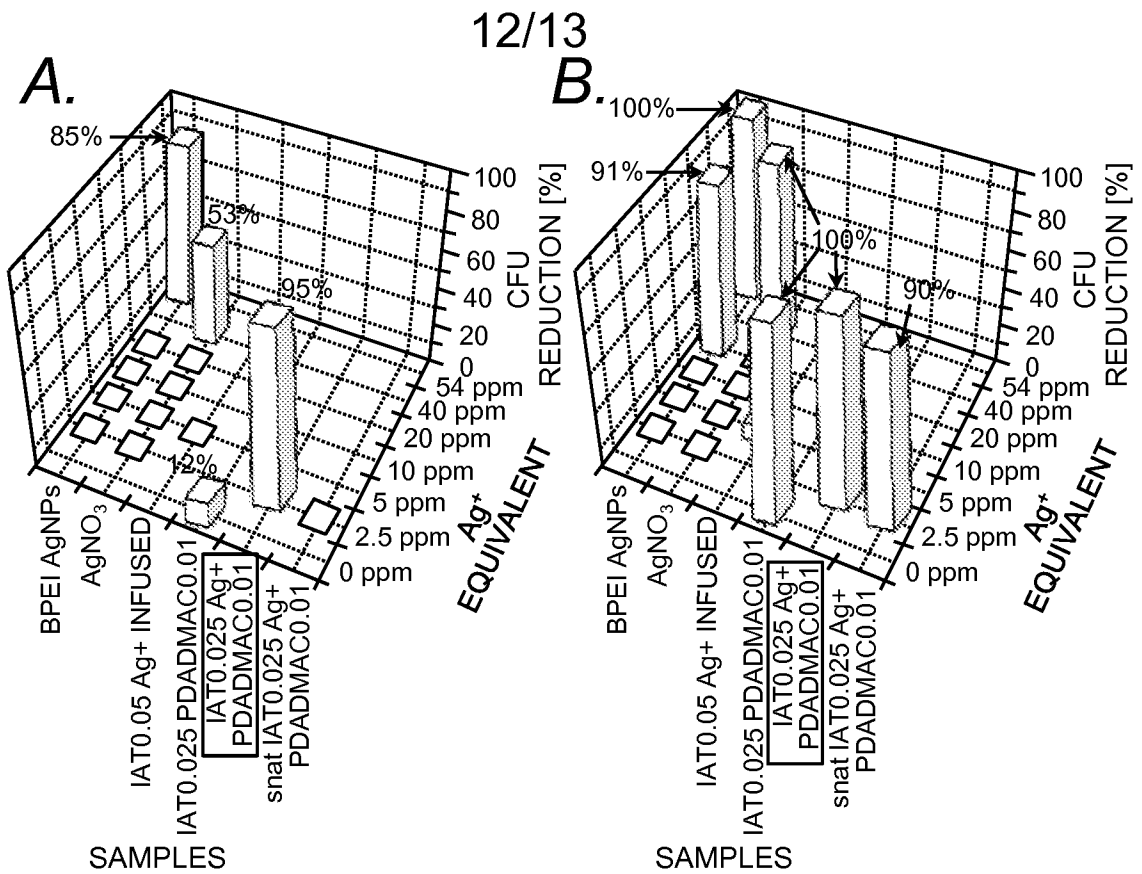


Figure 21

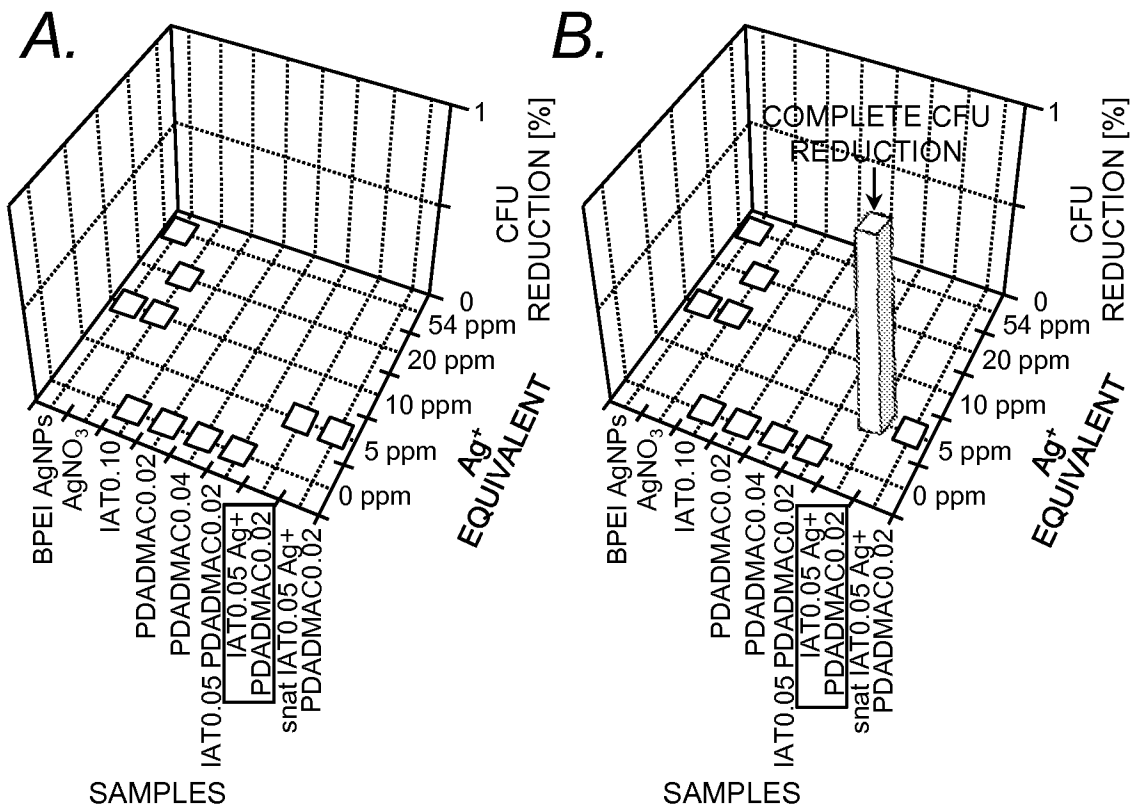
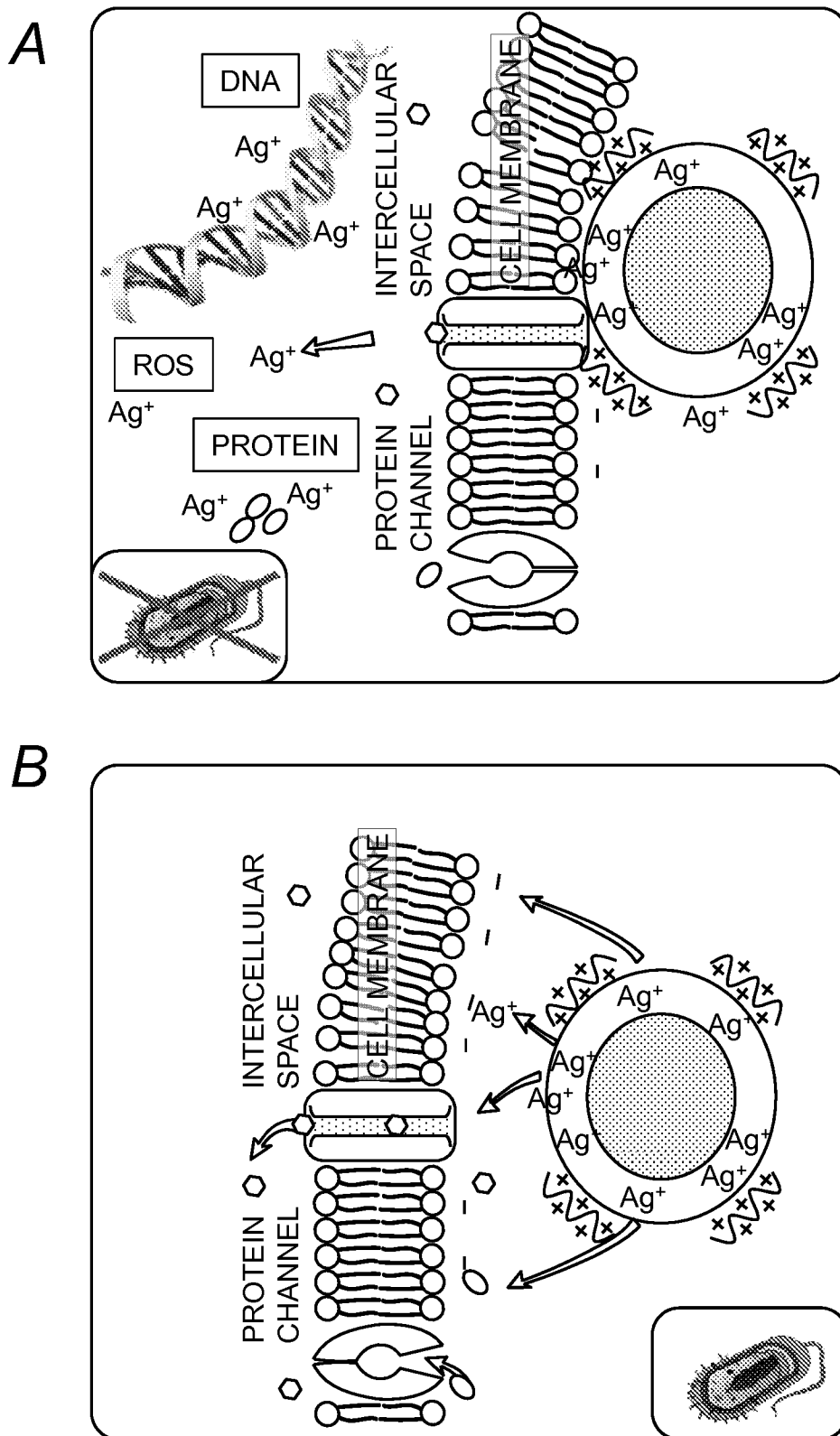


Figure 22

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*Figure 23*

INTERNATIONAL SEARCH REPORT

014/022382 18.06.2014

International application No.

PCT/US 14/22382

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C09D 101/18 (2014.01)

USPC - 524/31,35;525/54.21

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)

USPC (524/31; 524/35; 525/54.21; 424/489; 514/11.3; 514/18.6; 514/5.9; 514/56; 514/8.1; 514/8.2; 514/8.5; 514/8.9; 514/9.1; 514/9.6; 604/500; 424/401; 428/365; 428/327; 428/323; 424/492; 536/20; 436/163; 507/201; 504/103)

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

USPC (524/31; 524/35; 525/54.21; 424/489; 514/11.3; 514/18.6; 514/5.9; 514/56; 514/8.1; 514/8.2; 514/8.5; 514/8.9; 514/9.1; 514/9.6; 604/500; 424/401; 428/365; 428/327; 428/323; 424/492; 536/20; 436/163; 507/201; 504/103) – keyword delimited

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

PatBase, Google Patents/Scholar - Terms: nanoparticle core shell coat biodegradable biopolymer lignin chitosan derivative solvent anti-solvent pH alter antiviral cytotoxic cellulose oil fungicide biocide captan zein sulfonated imazomox agriculture surfactant decompose humic acid soy protein encapsulate layer nanorod nanofiber nanowire nanobelt

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2007/0243259 A1 (SUNG et al.) 18 Oct 2007 (18.10.2007) para [0004], [0009], [0010], [0012], [0035], [0043], [0063], [0064], [0072], [0095]; abstract; figure 4	1, 3, 9, 33, 34, 36-39, 42-45, 70, 74 and 75
Y		2, 4-8, 10-32, 35, 40-41, 46-69 and 71-73
Y	US 2012/0190593 A1 (SOANE et al.) 26 July 2012 (26.07.2012) para [0024], [0029], [0032], [0034], [0056], [0084]; figure 1A	2,4-8, 54-56 and 59-61
Y	US 2012/0195947 A1 (PERUMAL et al.) 2 Aug 2012 (02.08.2012) para [0003], [0010], [0081], [0088], [0114], [0115], [0151], [0152], [0155], [0157], [0165], [0173], [0192], [0193], [0198], [0248]; claim 1	10-32, 40, 41, 47-48, 51-52, 54-56, 62-64 and 71-73
Y	US 2004/0115424 A1 (COWTON) 17 June 2004 (17.06.2004) abstract; para [0025], [0026], [0105]	35 and 56
Y	US 2010/0247908 A1 (VELEV et al.) 30 Sep 2010 (30.09.2010) para [0025], [0030], [0031], [0036], [0044]; claim 1	46 and 50

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

* Special categories of cited documents:

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

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

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Date of the actual completion of the international search

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2005/0226938 A1 (BORBELY et al.) 13 Oct 2005 (13.10.2005) para [0011]-[0013]	49 and 53
Y	WO 2007/092029 A2 (HATTON et al.) 16 Aug 2007 (16.08.2007) pg 26, ln 3-6 and 21-23; claim 1; abstract	57 and 58
Y	US 5,919,487 A (SIMONNET et al.) 6 July 1999 (06.07.1999) col 1, ln 51-60; col 2, ln 49-67; col 6, ln 25-40	65 and 66
Y	WO 2012/140252 A1 (SALMAN et al.) 18 Oct 2012 (18.10.2012) pg 18, ln 19-25; pg 22, ln 1-5; pg 36, ln 16-32	15, 67 and 68
Y	US 2011/0263037 A1 (HERZ et al.) 27 Oct 2011 (27.10.2011) abstract; para [0008], [0075], [0180]	69
Y	US 2010/0056399 A1 (BERKLAND et al.) 4 March 2010 (04.03.2010) para [0030], [0036]	8
Y	US 2011/0111957 A1 (ISHAQUE et al.) 12 May 2011 (12.05.2011) para [0107], [0108], [0113], [0114]; claim 16	21, 23, 24 and 26
Y	WO 2012/058627 A2 (ZHANG et al.) 3 May 2012 (03.05.2012) pg 8, ln 29-33	32
Y	US 2011/0257006 A2 (THIEULEUX et al.) 20 Oct 2011 (20.10.2011) abstract; para [0077]	71