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(54) Title: THERAPEUTIC PROTEIN-BASED NANOPARTICLES AND METHODS FOR MAKING THE SAME

(57) Abstract: Protein-based nanoparticles and methods of forming such protein-based nanoparticles via electrohydrodynamic jetting methods are provided. The nanoparticle may comprise a water-soluble protein having an average molecular weight of $\geq$ about 8 kDa and $\leq$ about 700 kDa. In certain variations, the water-soluble protein is cross-linked (e.g., with an optional crosslinking agent) and defines a mesh structure having an average linear mesh size of $\geq$ about 1 nm to $\leq$ about 4 nm. Methods of making such nanoparticles may include jetting a liquid comprising the water-soluble protein through a nozzle, followed by exposing the liquid to an electric field sufficient to solidify the liquid and form the protein-based nanoparticles described above.



THERAPEUTIC PROTEIN-BASED NANOPARTICLES AND METHODS FOR MAKING THE SAME

CROSS-REFERENCE TO RELATED APPLICATIONS

5 [0001] This application claims the benefit of U.S. Provisional Application No. 62/931,512, filed on November 6, 2019. The entire disclosure of the above application is incorporated herein by reference.

FIELD

[0002] The present disclosure relates to therapeutic nanoparticles comprising at least one cross-linked water-soluble protein and electrohydrodynamic jetting methods for making the same.

BACKGROUND

10 [0003] This section provides background information related to the present disclosure which is not necessarily prior art.

[0004] Clinical translation of nanoparticle-based drug delivery systems is hindered by an array of challenges including poor circulation time and limited targeting.

15 [0005] In recent decades, many nanoparticle technologies have been developed for use in a variety medical applications. Generally, nanoparticles may be considered to be colloidal particles ranging in size from nanometers into the submicron range. However, the scope of their properties, modes of preparation, compositions, and architectures vary vastly. Such nanoparticles can be used in numerous biomedical applications including drug delivery, cancer therapy (e.g., glioblastoma),
20 tissue engineering, medical imaging and diagnostics, and immunotherapy.

[0006] There remains a need in the field for development of improved nanoparticle-based drug delivery systems aimed to provide: (i) protection of loaded cargo from degradation or deactivation, (ii) potential controlled release mechanisms, and (iii) altered pharmacokinetics and specific control of biodistribution.

SUMMARY

25 [0007] This section provides a general summary of the disclosure, and is not a comprehensive disclosure of its full scope or all of its features.

[0008] The present disclosure relates to therapeutic protein-based nanoparticles and methods for making the protein-based nanoparticles.

30 [0009] In various aspects, the current technology provides a nanoparticle including a cross-linked water-soluble protein having a mesh structure, wherein the water-soluble protein has

an average molecular weight of greater than or equal to about 8 kDa and less than or equal to about 700 kDa.

[0010] In one aspect, the mesh structure has an average linear mesh size of greater than or equal to about 1 nm to less than or equal to about 4 nm.

5 [0011] In one aspect, the nanoparticle includes a crosslinking agent conjugated to the water-soluble protein.

[0012] In one aspect, the cross-linked water soluble protein is present at greater than or equal to about 50% by weight to less than or equal to about 95% by weight, and wherein the crosslinking agent is present at greater than or equal to about 5% by weight to less than or equal
10 to about 50% by weight.

[0013] In one aspect, prior to reacting with the water-soluble protein, the crosslinking agent includes a reactive group selected from the group consisting of an alkenyl group, an alkynyl group, a maleimide group, an active ester group, an anhydride group, an N-succinimidyl group, a triflate group, and a combination thereof.

15 [0014] In one aspect, the crosslinking agent is a homo-bifunctional polymer.

[0015] In one aspect, the nanoparticle further includes one or more of a therapeutic active ingredient, an imaging agent, and a targeting moiety.

[0016] In one aspect, the nanoparticle includes a therapeutic active ingredient which is a biomolecule.

20 [0017] In one aspect, the biomolecule is a nucleic acid.

[0018] In one aspect, the biomolecule is DNA.

[0019] In one aspect, the water-soluble protein is selected from the group consisting of albumin, ovalbumin, mucin, transferrin, insulin, lysozyme, hemoglobin, collagen, and a combination thereof.

25 [0020] In various aspects, the current technology also provides a method of treating a subject having a cancer, the method including administering to the subject the nanoparticle in an effective amount to treat the cancer.

[0021] In various aspects, the current technology further provides a pharmaceutical composition including the nanoparticle.

30 [0022] In various aspects, the current technology yet further provides a method of making a nanoparticle, the method including jetting a liquid including a water-soluble protein having an average molecular weight of greater than or equal to about 8 kDa and less than or equal to about 700 kDa and water through a nozzle; and exposing the liquid to an electric field sufficient to solidify the liquid and form the nanoparticle defining a mesh structure having an average linear
35 mesh size of greater than or equal to about 1 nm to less than or equal to about 4 nm.

[0023] In one aspect, the liquid further includes a crosslinking agent and during the exposing, the water-soluble protein is at least partially cross-linked.

5 [0024] In one aspect, the at least partially cross-linked water-soluble protein defines a mesh structure having an average linear mesh size of greater than or equal to about 1 nm to less than or equal to about 4 nm.

[0025] In one aspect, the electric field is formed by applying a potential difference between at least two electrodes from about 0.1 kV to about 25 kV.

10 [0026] In one aspect, the liquid further includes an additive selected from the group consisting of a therapeutic active ingredient, an imaging agent, a targeting moiety, and a combination thereof, wherein the additive is incorporated into the nanoparticle.

[0027] In one aspect, the additive is a therapeutic active ingredient which is a biomolecule.

[0028] In one aspect, the biomolecule is a nucleic acid.

[0029] In one aspect, the biomolecule is DNA.

15 [0030] In various aspects, the current technology also provides a nanoparticle including a cross-linked water-soluble protein having an average molecular weight of greater than or equal to about 8 kDa to less than or equal to about 700 kDa and having disulfide bonds, wherein the nanoparticle is substantially free of a distinct crosslinking agent.

[0031] In one aspect, the cross-linked water-soluble protein defines a mesh structure.

20 [0032] In one aspect, the cross-linked water-soluble protein is selected from the group consisting of albumin, human serum albumin, ovalbumin, bovine serum albumin, transferrin, hemoglobin, IgG, enzymes, transport proteins, storage proteins, antibodies, aptamers, chemokines, hormonal proteins, polypeptides, and combinations thereof.

[0033] In one aspect, the nanoparticle further includes one or more of a therapeutic active ingredient, an imaging agent, a biomolecule, and a targeting moiety.

25 [0034] In various aspects, the current technology provides a method of treating a subject having a cancer, the method including administering to the subject the nanoparticle in an effective amount to treat the cancer.

[0035] In various aspects, the current technology further provides a pharmaceutical composition including the nanoparticle.

30 [0036] In various aspects, the current technology yet further provides a method of making a nanoparticle, the method including jetting a liquid including a water-soluble protein having an average molecular weight of greater than or equal to about 8 kDa to less than or equal to about 700 kDa and including disulfide bonds through a nozzle; and exposing the liquid to an electric field sufficient to cross-link and solidify the liquid and form the nanoparticle that is substantially
35 free of a distinct crosslinking agent.

[0037] In one aspect, the water-soluble protein defines a mesh structure having an average linear mesh size of greater than or equal to about 1 nm to less than or equal to about 4 nm.

[0038] In one aspect, the electric field is formed by applying a potential difference between at least two electrodes from about 0.1 kV to about 25 kV.

5 [0039] In one aspect, the liquid further includes an additive selected from the group consisting of a therapeutic active ingredient, an imaging agent, a targeting moiety, and a combination thereof, wherein the additive is incorporated into the nanoparticle.

[0040] In one aspect, the therapeutic active ingredient is a biomolecule.

[0041] In one aspect, the biomolecule is a nucleic acid.

10 [0042] In one aspect, the biomolecule is DNA.

[0043] In one aspect, the water-soluble protein is selected from the group consisting of albumin, human serum albumin, ovalbumin, bovine serum albumin, transferrin, hemoglobin, IgG, an enzyme, a transport protein, a storage protein, an antibody, an aptamer, a chemokines, a hormonal protein, a polypeptide, and a combination thereof.

15 [0044] In various aspects, the current technology provides a multicompartmental nanoparticle including a first compartment defining at least a portion of an exposed surface of the multicompartmental nanoparticle and including a first composition having a water-soluble polymer having an average molecular weight of greater than or equal to about 8 kDa and less than or equal to about 700 kDa; and at least one additional compartment defining at least a portion of an exposed
20 surface and including at least one additional composition distinct from the first composition.

[0045] In one aspect, the multicompartmental nanoparticle further includes a crosslinking agent conjugated to the water-soluble protein in the first compartment.

[0046] In one aspect, the water-soluble protein is present at greater than or equal to about 50% by weight to less than or equal to about 95% by weight and the crosslinking agent is present
25 at greater than or equal to about 5% by weight to less than or equal to about 50% by weight.

[0047] In one aspect, prior to reacting with the water-soluble protein, the crosslinking agent includes a reactive group selected from the group consisting of an alkenyl group, an alkynyl group, a maleimide group, an active ester group, an anhydride group, an N-succinimidyl group, a triflate group, and combinations thereof.

30 [0048] In one aspect, the multicompartmental nanoparticle further includes one or more of a therapeutic active ingredient, an imaging agent, and a targeting moiety.

[0049] In one aspect, the multicompartmental nanoparticle includes a therapeutic active ingredient which is a biomolecule.

[0050] In one aspect, the biomolecule is a nucleic acid.

35 [0051] In one aspect, the biomolecule is DNA.

[0052] In one aspect, the water-soluble protein is selected from the group consisting of albumin, ovalbumin, mucin, transferrin, insulin, lysozyme, hemoglobin, collagen, and a combination thereof.

5 [0053] In one aspect, the water-soluble protein is a first water soluble protein and the at least one additional compartment includes a second water-soluble protein.

[0054] In one aspect, the second water-soluble protein is selected from the group consisting of albumin, ovalbumin, mucin, transferrin, insulin, lysozyme, hemoglobin, collagen, and a combination thereof.

10 [0055] In various aspects, the current technology further provides a method of treating a subject having a cancer, the method including administering to the subject the multicompartmental nanoparticle in an effective amount to treat the cancer.

[0056] In various aspects, the current technology yet further provides a pharmaceutical composition including the multicompartmental nanoparticle.

15 [0057] In various aspects, the current technology provides a nanoparticle including a cross-linked water-soluble protein having an average molecular weight of greater than or equal to about 8 KDa and less than or equal to about 700 kDa and a therapeutic active ingredient.

[0058] In one aspect, the therapeutic active ingredient is selected from the group consisting of DNA, RNA, plasmids, short interfering sequence of double stranded RNA (siRNA), messenger RNA (mRNA), transfer RNA, ribosomal RNA, small nuclear RNA, single stranded DNA, CRISPR
20 CAS-9, aptamers, antibodies, peptides, targeting molecules, vitamins, and combinations thereof.

[0059] In one aspect, the nanoparticle further includes a crosslinking agent conjugated to the water-soluble protein.

25 [0060] In one aspect, the cross-linked water soluble protein is present at greater than or equal to about 50% by weight to less than or equal to about 95% by weight and the crosslinking agent is present at greater than or equal to about 5% by weight to less than or equal to about 50% by weight.

[0061] In one aspect, prior to reacting with the water-soluble protein, the crosslinking agent includes a reactive group selected from the group consisting of an alkenyl group, an alkynyl group, a maleimide group, an active ester group, an anhydride group, an N-succinimidyl group, a triflate group, and a combination thereof.

30 [0062] In one aspect, the nanoparticle further includes one or more of an imaging agent, an additional biomolecule, and a targeting moiety.

[0063] In one aspect, the therapeutic active ingredient is selected from the group consisting of a drug, a steroid, and combinations thereof.

35 [0064] In one aspect, the drug is selected from the group consisting of: paclitaxel, cis-platin, doxorubicin, and combinations thereof.

[0065] In one aspect, the therapeutic active ingredient is selected from the group consisting of: an antibody, an aptamer, a chemokine, a peptide drug, and combinations thereof.

[0066] In one aspect, the water-soluble protein is selected from the group consisting of albumin, ovalbumin, mucin, transferrin, insulin, lysozyme, hemoglobin, collagen, and a combination thereof.

[0067] Further areas of applicability will become apparent from the description provided herein. The description and specific examples in this summary are intended for purposes of illustration only and are not intended to limit the scope of the present disclosure.

DRAWINGS

[0068] The drawings described herein are for illustrative purposes only of selected embodiments and not all possible implementations, and are not intended to limit the scope of the present disclosure.

[0069] FIG. 1 shows a cross-section view of an example of a nanoparticle formed of water-soluble proteins according to certain aspects of the present disclosure.

[0070] FIG. 2 shows a detailed view of a nanoparticle having cross-linked proteins defining a representative mesh structure according to certain aspects of the present disclosure.

[0071] FIG. 3 shows an example of a schematic of an electrohydrodynamic jetting system for forming nanoparticles comprising a water-soluble protein in accordance with certain aspects of the present disclosure.

[0072] FIG. 4 shows another example of a schematic of an electrohydrodynamic jetting system for co-jetting and forming multicompartmental nanoparticles comprising water-soluble protein in at least one compartment in accordance with certain aspects of the present disclosure.

[0073] FIGS. 5A-5D show SEM images of different ovalbumin nanoparticles formed via an electrohydrodynamic jetting process in accordance with certain aspects of the present disclosure. FIG. 5A shows an ovalbumin nanoparticle with 10 w/w% NHS-PEG-NHS crosslinker (MW=2000 g/mol (2k XL)). FIG. 5B shows an ovalbumin nanoparticle with 30 w/w% 2k XL. FIG. 5C shows an ovalbumin nanoparticle with 50 w/w% 2k XL fabricated with 80:20 vol. % ethylene glycol/water. FIG. 5D shows 5 w/w% 20k XL fabricated with 40:60 vol. % ethylene glycol/water.

[0074] FIGS. 6A-6D. FIG. 6A shows size distributions of the ovalbumin (OVA) protein-based particles (pNPs) in FIGS. 5A-5D. The size distributions are obtained by measuring NP diameters using ImageJ. The size of hydrated pNPs is measured using DLS after NP collection and dispersion in PBS buffer. FIG. 6B is a table with parameters/conditions for electrospraying of the OVA pNPs. FIG. 6C is SANS data and fits for OVA pNPs with 10% and 50% XL. OVA pNPs are dispersed in D₂O at 2 mg/mL. Data are fitted using the Debye-Anderson-Brumberger (DAB)

model. FIG. 6D shows measured Young's modulus as a function of the pNP crosslinker amount. Data are obtained by fitting the force-distance profiles obtained from AFM measurements using the Hertz model for a conical indenter.

[0075] FIGS. 7A–7G. FIG. 7A shows uptake of fluorescently labeled ovalbumin (OVA) protein-based particles (pNPs) by bone marrow-derived dendritic cells (BMDCs). FIGS. 7B–7E show quantitative uptake data (MFI values) obtained by flow cytometry for ovalbumin nanoparticles with 5 w/w% NHS-PEG-NHS crosslinker, 10 w/w% NHS-PEG-NHS crosslinker, 30 w/w% NHS-PEG-NHS crosslinker, and 50% w/w% NHS-PEG-NHS crosslinker. Uptake is further visualized by confocal microscopy. BMDCs are incubated with OVA pNPs (10 μ g/mL) for 24 hours. For flow cytometry, BMDCs are stained for DC marker CD11c+ using anti-CD11c+ PE-Cy7; they are also stained with DAPI. For confocal microscopy, actin is stained with phalloidin488 and nuclei are stained with DAPI. The data represent the mean $\pm$ SEM from triplicates of 3 experiments. The data are analyzed by one-way ANOVA, followed by Tukey's post-test, using GraphPad 6.0. A P-value of <0.05 is considered statistically significant (* $P<0.05$, ** $P<0.01$, *** $P<0.001$; **** $P<0.0001$); P-values of >0.05 are considered not significant (ns). FIG. 7F shows OVA pNP-treated BMDCs induce proliferation of OT-I CD8+ cells. A percentage of proliferated OT-I CD8+ cells after co-culture with BMDCs incubated with 10 μ g/mL OVA pNPs (5% 20k XL, 10% 2k XL, 30% 2k XL, 50% 2k XL) are shown. FIG. 7G shows representative flow cytometry histograms of FIG. 7F. The data represent the mean $\pm$ SEM from triplicates of three experiments. The data are analyzed by linear-mixed model with Tukey's post-test using RStudio software. A P-value of <0.05 is considered statistically significant (* $P<0.05$, ** $P<0.01$, *** $P<0.001$; **** $P<0.0001$); P-values of >0.05 are considered not significant (ns).

[0076] FIGS. 8A–8F. FIGS. 8A–8F show humoral immune responses elicited by polymerized OVA pNPs in vivo. FIG. 8A show vaccine doses and regimen. Naïve C57BL/6 mice are injected with OVA pNPs and soluble CpG subcutaneously at the tail base on Day 0 (prime immunization) and 21 (boost immunization). Data are analyzed using multiple t-test. $P < 0.05$ is considered statistically different (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$); $P > 0.05$ is considered not significant. Serum anti-OVA IgG titers are measured on day 20 (shown in FIG. 8B – prime response) and day 28 (FIG. 8C – boost response). The data are fitted by logarithmic regression. The titer is calculated by solving for the inverse dilution factor resulting in an absorbance value of 0.5. Data represent mean $\pm$ SEM (n=5). Groups are compared using one-way ANOVA with Tukey's post-test. $P < 0.05$ is considered statistically different (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$). $P > 0.05$ is considered not significant. Delivery of pNPs to dLNs: MFI of AlexaFluor 647 associated with OVA NPs among (FIG. 8D) F4/80+ macrophages, (FIG. 8E) B220+ B cells and (FIG. 8F) CD11c+ DCs obtained from a single cell suspension from draining lymph

nodes. Groups are compared using one-way ANOVA with Tukey's post-test. $P < 0.05$ is considered statistically different (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$). $P > 0.05$ is considered not significant.

[0077] FIGS. 9A–9B. FIGS. 9A–9B show therapeutic effect of OVA pNPs. In FIG. 9A, a vaccine doses and regimen for a murine model is shown. FIG. 9B shows animal survival. C57BL/6 mice are inoculated subcutaneously with 1×10^5 B16F10-OVA cells on day 0. On days 7 and 14, mice are treated with indicated formulations (OVA pNP, soluble OVA, PBS) containing 10 $\mu\text{g}/\text{dose}$ OVA and 15 $\mu\text{g}/\text{dose}$ CpG (100 μL dose). Data represent mean $\pm$ SEM ($n=10$). Groups are compared using Kaplan-Meier estimator analysis. $P < 0.05$ is considered statistically different (* $P < 0.05$). $P > 0.05$ is considered not significant.

[0078] Corresponding reference numerals indicate corresponding parts throughout the several views of the drawings.

DETAILED DESCRIPTION

[0079] Example embodiments are provided so that this disclosure will be thorough, and will fully convey the scope to those who are skilled in the art. Numerous specific details are set forth such as examples of specific compositions, components, devices, and methods, to provide a thorough understanding of embodiments of the present disclosure. It will be apparent to those skilled in the art that specific details need not be employed, that example embodiments may be embodied in many different forms and that neither should be construed to limit the scope of the disclosure. In some example embodiments, well-known processes, well-known device structures, and well-known technologies are not described in detail.

[0080] The terminology used herein is for the purpose of describing particular example embodiments only and is not intended to be limiting. As used herein, the singular forms “a,” “an,” and “the” may be intended to include the plural forms as well, unless the context clearly indicates otherwise. The terms “comprises,” “comprising,” “including,” and “having,” are inclusive and therefore specify the presence of stated features, elements, compositions, steps, integers, operations, and/or components, but do not preclude the presence or addition of one or more other features, integers, steps, operations, elements, components, and/or groups thereof. Although the open-ended term “comprising,” is to be understood as a non-restrictive term used to describe and claim various embodiments set forth herein, in certain aspects, the term may alternatively be understood to instead be a more limiting and restrictive term, such as “consisting of” or “consisting essentially of.” Thus, for any given embodiment reciting compositions, materials, components, elements, features, integers, operations, and/or process steps, the present disclosure also specifically includes embodiments consisting of, or consisting essentially of, such recited compositions, materials, components, elements, features, integers, operations, and/or process

steps. In the case of “consisting of,” the alternative embodiment excludes any additional compositions, materials, components, elements, features, integers, operations, and/or process steps, while in the case of “consisting essentially of,” any additional compositions, materials, components, elements, features, integers, operations, and/or process steps that materially affect the basic and novel characteristics are excluded from such an embodiment, but any compositions, materials, components, elements, features, integers, operations, and/or process steps that do not materially affect the basic and novel characteristics can be included in the embodiment.

[0081] Any method steps, processes, and operations described herein are not to be construed as necessarily requiring their performance in the particular order discussed or illustrated, unless specifically identified as an order of performance. It is also to be understood that additional or alternative steps may be employed, unless otherwise indicated.

[0082] When a component, element, or layer is referred to as being “on,” “engaged to,” “connected to,” or “coupled to” another element or layer, it may be directly on, engaged, connected or coupled to the other component, element, or layer, or intervening elements or layers may be present. In contrast, when an element is referred to as being “directly on,” “directly engaged to,” “directly connected to,” or “directly coupled to” another element or layer, there may be no intervening elements or layers present. Other words used to describe the relationship between elements should be interpreted in a like fashion (*e.g.*, “between” versus “directly between,” “adjacent” versus “directly adjacent,” etc.). As used herein, the term “and/or” includes any and all combinations of one or more of the associated listed items.

[0083] Although the terms first, second, third, etc. may be used herein to describe various steps, elements, components, regions, layers and/or sections, these steps, elements, components, regions, layers and/or sections should not be limited by these terms, unless otherwise indicated. These terms may be only used to distinguish one step, element, component, region, layer or section from another step, element, component, region, layer or section. Terms such as “first,” “second,” and other numerical terms when used herein do not imply a sequence or order unless clearly indicated by the context. Thus, a first step, element, component, region, layer or section discussed below could be termed a second step, element, component, region, layer or section without departing from the teachings of the example embodiments.

[0084] Spatially or temporally relative terms, such as “before,” “after,” “inner,” “outer,” “beneath,” “below,” “lower,” “above,” “upper,” and the like, may be used herein for ease of description to describe one element or feature's relationship to another element(s) or feature(s) as illustrated in the figures. Spatially or temporally relative terms may be intended to encompass different orientations of the device or system in use or operation in addition to the orientation depicted in the figures.

[0085] Throughout this disclosure, the numerical values represent approximate measures or limits to ranges to encompass minor deviations from the given values and embodiments having about the value mentioned as well as those having exactly the value mentioned. Other than in the working examples provided at the end of the detailed description, all numerical values of parameters (e.g., of quantities or conditions) in this specification, including the appended claims, are to be understood as being modified in all instances by the term “about” whether or not “about” actually appears before the numerical value. “About” indicates that the stated numerical value allows some slight imprecision (with some approach to exactness in the value; approximately or reasonably close to the value; nearly). If the imprecision provided by “about” is not otherwise understood in the art with this ordinary meaning, then “about” as used herein indicates at least variations that may arise from ordinary methods of measuring and using such parameters. For example, “about” may comprise a variation of less than or equal to 5%, optionally less than or equal to 4%, optionally less than or equal to 3%, optionally less than or equal to 2%, optionally less than or equal to 1%, optionally less than or equal to 0.5%, and in certain aspects, optionally less than or equal to 0.1%.

[0086] In addition, disclosure of ranges includes disclosure of all values and further divided ranges within the entire range, including endpoints and sub-ranges given for the ranges.

[0087] Example embodiments will now be described more fully with reference to the accompanying drawings.

[0088] In various aspects, the present disclosure provides a nanoparticle formed of a protein. For example, a representative view of one such a nanoparticle 20 in the form of a sphere is shown in FIG. 1. The nanoparticle 20 includes a plurality of proteins 22 that are cross-linked and together define the nanoparticle 20. The nanoparticle 20 may have a diameter “D” or major dimension that is in the nanoscale range.

[0089] A nanoparticle is a material that has a variety of shapes or morphologies, however, generally has at least one spatial dimension that is less than about 1 μm (i.e., 1,000 nm), optionally less than about 0.75 μm (i.e., 750 nm), optionally less than about 0.5 μm (i.e., 500 nm), and in certain aspects, less than about 0.25 μm (i.e., 200 nm). In some instances, the nanoparticle has a least one spatial dimension that is less than about 300 nm (e.g., diameter of less than 300 nm (e.g., mean diameter of less than 300 nm or median diameter of less than 300 nm), e.g., diameter between 100 nm and 300 nm (e.g., mean diameter between 100 nm and 300 nm or median diameter between 100 nm and 300 nm), e.g., from 100 to 150 nm, from 150 to 200 nm, from 200 to 250 nm, or from 250 to 300 nm, e.g., about 150 nm, about 200 nm, about 250 nm, or about 300 nm). In some instances, the nanoparticle has at least one spatial dimension that is less than about 100 nm (e.g., diameter of less than 100 nm (e.g., mean diameter of less than 100 nm or median diameter of less than 100 nm), e.g., diameter between 10 nm and 100 nm (e.g., mean diameter

between 10 nm and 100 nm or median diameter between 10 nm and 100 nm), e.g., about 95 nm, about 90 nm, about 85 nm, about 80 nm, about 75 nm, about 70 nm, about 65 nm, about 60 nm, about 55 nm, about 50 nm, or about 40 nm (e.g., mean diameter of about 95 nm, about 90 nm, about 85 nm, about 80 nm, about 75 nm, about 70 nm, about 65 nm, about 60 nm, about 55 nm, about 50 nm, or about 40 nm or median diameter of about 95 nm, about 90 nm, about 85 nm, about 80 nm, about 75 nm, about 70 nm, about 65 nm, about 60 nm, about 55 nm, about 50 nm, or about 40 nm)). In certain aspects, a nanoparticle has at least one spatial dimension, such as a diameter, that is greater than or equal to about 5 nm and less than or equal to about 1,000 nm, optionally greater than or equal to about 5 nm to less than or equal to about 500 nm, optionally greater than or equal to about 10 nm to less than or equal to about 1,000 nm, optionally greater than or equal to about 10 nm to less than or equal to about 500 nm, optionally greater than or equal to about 25 nm to less than or equal to about 1,000 nm, optionally greater than or equal to about 25 nm to less than or equal to about 500 nm, optionally greater than or equal to about 50 nm to less than or equal to about 1,000 nm, optionally greater than or equal to about 50 nm to less than or equal to about 500 nm, optionally greater than or equal to about 100 nm to less than or equal to about 1,000 nm, and in certain aspects, optionally greater than or equal to about 100 nm to less than or equal to about 500 nm.

[0090] The nanoparticle may have a variety of geometries or morphologies, including, by way of non-limiting example, substantially round shapes, like spheres and ellipsoids/ovals, rectangles, polygons, discoids/discs, ellipsoids, toroids, cones, pyramids, rods/cylinders, and the like. In certain aspects, the nanoparticle may have a substantially round shape, such as spheres, ellipsoids, hemispheres, and the like.

[0091] In certain variations, the nanoparticle comprises a water-soluble protein that may be cross-linked. The water-soluble protein may have an average molecular weight of greater than or equal to about 8 kDa and less than or equal to about 700 kDa, so long as it is water soluble. In certain variations, the cross-linked water-soluble protein nanoparticles define a caged or mesh structure. By mesh structure, it is meant that the nanoparticle has a cross-linked protein network that defines three-dimensional structures having openings, voids, or pores defined therein. The mesh size is one defining aspect of the protein nanoparticles according to the present teachings.

[0092] For purposes of illustration, FIG. 2 shows a detailed view of a representative non-limiting mesh structure 50 within a nanoparticle comprising a cross-linked water-soluble protein. While the nanoparticle may be considered to be porous, in contrast to more traditional porous materials, the mesh structure may be considered to be a more open structure with a high porosity level so that a large portion of the nanoparticle comprises openings defined between each pore. Further, the openings are interconnected with one another. In other words, the cross-linked protein defines a cage-like or structural lattice-type framework that may be considered to be

interconnected struts and bridges that create the mesh structure 50. In certain aspects, the mesh structure 50 may have an overall open volume (aside from the water-soluble proteins and optional crosslinking agents) of greater than or equal to about 50 volume %, optionally greater than or equal to about 60 volume %, optionally greater than or equal to about 70 volume %, and optionally greater than or equal to about 80 volume % of the overall nanoparticle volume.

[0093] FIG. 2 shows one layer of the mesh structure 50 in a nanoparticle prepared in accordance with certain aspects of the present disclosure. A plurality of proteins 52 are connected by cross-linking bridges 54. In certain variations, the cross-linking bridges 54 may be distinct crosslinking agents reacted with (*e.g.*, conjugated to) the water-soluble protein. In other variations, the water-soluble proteins may be self-cross-linked without any distinct crosslinking agent present. As shown in FIG. 2, an opening 60 is defined between a first crosslink bridge 70, a first protein 80, a second crosslink bridge 72, a second protein 82, a third crosslink bridge 74, a third protein 84, a fourth crosslink bridge 76, and a fourth protein 86. As can be seen, the first crosslink bridge 70 is reacted with and links the first protein 80 and second protein 82. The second crosslink bridge 72 links the second protein 82 and third protein 84. The third crosslink bridge 74 links the third protein 84 and fourth protein 86. Finally, the fourth crosslink bridge 76 links the fourth protein 86 and the first protein 80. As will be appreciated by those of skill in the art, the various proteins and bridges may extend in other directions to form a three-dimensional structure or mesh framework. The mesh structure 50 thus has a plurality of interior voids or openings 60 that have a representative rectangular shape in a regular repeating three-dimensional mesh pattern; however, the openings 60 are not necessarily limited to these shapes or positions and may have different shapes and arrangements within the mesh structure 50.

[0094] The openings 60 within the mesh structure 50 may be understood to have an average mesh size. Mesh size (ξ), also referred to as correlation length, can be understood to represent a maximum size of solutes/molecules that can pass through the mesh structure 50, where the dimensions of such a molecule are labeled 62 in FIG. 2. In certain aspects, an average linear mesh size (ξ) (*e.g.*, represented by dimension 62) in the mesh structure 50 may be greater than or equal to about 1 nm to less than or equal to about 4 nm (*e.g.*, from 1 nm to 3 nm, or from 2 nm to 4 nm, *e.g.*, from 1 nm to 2 nm, from 2 nm to 3 nm, or from 3 nm to 4 nm, *e.g.*, about 1 nm, about 2 nm, about 3 nm, or about 4 nm). Inside the openings 60 in the hollow cage-like mesh structure 50, it is possible to load a variety of therapeutic molecules, such as therapeutic active ingredients, biomolecules (*e.g.*, nucleic acids (*e.g.*, DNA, RNA) and/or proteins), enzymes, small molecules, imaging agents, and the like.

[0095] Thus, the average mesh size within protein nanoparticles is an important factor affecting controlled release of therapeutic agents or imaging agents. As will be described further

below, the methods of electrohydrodynamic jetting used to fabricate polymerized protein nanoparticles permits tuning a crosslinking density, for example, by simply changing a ratio of crosslinking agent to protein in the particle formulation solution step without the need for any further post-fabrication modification. By way of non-limiting example, electrohydrodynamic jetting enables fine-tuning of mesh sizes of protein-based nanoparticles by simply changing the ratio of protein to crosslinking agent. In the case of ovalbumin, where the ratio of crosslinking agent to protein is increased from 10% to 50%, the mesh size in the nanoparticle (length scale, average spacing ξ) decreases from about 3.98 ± 0.12 nm to about 2.15 ± 0.01 nm. In certain aspects, the cross-linked water-soluble protein is present at greater than or equal to about 50% by weight to less than or equal to about 95% by weight (*e.g.*, about 50% by weight, about 55% by weight, about 60% by weight, about 65% by weight, about 70% by weight, about 75% by weight, about 80% by weight, about 85% by weight, about 90% by weight, or about 95% by weight (*e.g.*, dry weight) of the nanoparticle) and the crosslinking agent is present at greater than or equal to about 5% by weight to less than or equal to about 50% by weight (*e.g.*, about 5% by weight, about 10% by weight, about 15% by weight, about 20% by weight, about 25% by weight, about 30% by weight, about 35% by weight, about 40% by weight, about 45% by weight, or about 50% by weight (*e.g.*, dry weight) of the nanoparticle). In one variation, the cross-linked water-soluble protein is present at about 60% by weight and the crosslinking agent is present at about 40% by weight.

[0096] In some instances, a cross-linking agent providing a suitable mesh size has an average molecular weight from 100 kDa to 100,000 kDa (*e.g.*, from 500 kDa to 50,000 kDa, or from 1,000 kDa to 25,000 kDa, *e.g.*, from 100 kDa to 500 kDa, from 500 kDa to 1,000 kDa, from 1,000 kDa to 2,000 kDa, from 2,000 kDa to 5,000 kDa, from 5,000 kDa to 10,000 kDa, from 10,000 kDa to 20,000 kDa, from 20,000 kDa to 50,000 kDa, or from 50,000 kDa to 100,000 kDa, *e.g.*, about 2,000 kDa, about 5,000 kDa, about 8,000 kDa, about 10,000 kDa, or about 20,000 kDa). In some instances, a cross-linking agent providing a suitable mesh size is a linear polymer (*e.g.*, a bifunctional linear polymer) and has an average molecular weight from 100 kDa to 100,000 kDa (*e.g.*, from 500 kDa to 50,000 kDa, or from 1,000 kDa to 25,000 kDa, *e.g.*, from 100 kDa to 500 kDa, from 500 kDa to 1,000 kDa, from 1,000 kDa to 2,000 kDa, from 2,000 kDa to 5,000 kDa, from 5,000 kDa to 10,000 kDa, from 10,000 kDa to 20,000 kDa, from 20,000 kDa to 50,000 kDa, or from 50,000 kDa to 100,000 kDa, *e.g.*, about 2,000 kDa, about 5,000 kDa, about 8,000 kDa, about 10,000 kDa, or about 20,000 kDa). In some instances, the cross-linking agent providing a suitable mesh size is a linear polymer (*e.g.*, a bifunctional linear polymer) having an average molecule weight of about 2,000 kDa.

[0097] In certain variations, the nanoparticle comprises a water-soluble protein having an average molecular weight of greater than or equal to about 8 kDa to less than or equal to about 700

kDa, optionally greater than or equal to about 10 kDa to less than or equal to about 400 kDa. In some instances, the nanoparticle comprises a water-soluble protein having an average molecular weight from 8 kDa to 15 kDa, from 10 kDa to 20 kDa, from 15 kDa to 25 kDa, from 25 kDa to 50 kDa, from 50 kDa to 100 kDa, from 100 kDa to 200 kDa, from 200 kDa to 300 kDa, from 300 kDa to 400 kDa, from 400 kDa to 500 kDa, from 500 kDa to 600 kDa, or from 600 kDa to 700 kDa.

[0098] While a large variety of proteins can be used, in certain aspects, the protein is water-soluble (*e.g.*, the un-crosslinked protein is water-soluble). Thus, the protein may be dissolved in water or carriers that are aqueous solutions that may comprise predominantly water. In certain aspects, proteins that are excluded from suitable proteins include transmembrane proteins, polytopic proteins that aggregate and precipitate in water, and proteins with a very high molecular weight, *e.g.*, a molecular weight greater than 700 kDa, greater than or equal to about 750 kDa, or greater than or equal to about 800 kDa. In some instances, the protein of the nanoparticle is not laminin. In other instances, the protein is not fibronectin. In yet other instances, the protein is not laminin or fibronectin. In some instances, the protein of the nanoparticle is not a native matrix protein (*e.g.*, not a naturally occurring extracellular matrix protein). Further, in certain aspects, small proteins with molecular weights less than 8 kDa are avoided, such as hirudin, which is only made up of 65 amino acids and has a molecular weight of about 6.7 kDa.

[0099] In certain aspects, the water-soluble protein having the desired molecular weight is selected from the group consisting of: albumin, ovalbumin, mucin, transferrin, insulin, lysozyme, hemoglobin, collagen, and combinations thereof. It should be noted that some of these proteins have varying molecular weights, so the protein selected desirably has a molecular weight in the range discussed above to ensure capability to be electrohydrodynamically jetted in an aqueous liquid, as will be described further below.

[0100] In certain aspects, the nanoparticle may further comprise a crosslinking agent reacted with the water-soluble protein. Prior to reacting with the water-soluble protein, the crosslinking agent may comprise a reactive group selected from the group consisting of: an alkenyl group, an alkynyl group, a maleimide group, an active ester group, an anhydride group, an N-succinimidyl group, a triflate group, and combinations thereof. In one variation, the crosslinking agent may be an amine-reactive crosslinker comprising a polyethylene glycol. For example, the crosslinking agent may be a homo-bifunctional polyethylene glycol crosslinker, O'-bis[2-(N-Succinimidyl-succinylamino)ethyl] polyethylene glycol (NHS-PEG-NHS), where the PEG units formed amide bonds with amino groups, such as lysine residues, in the water-soluble protein.

[0101] In certain other variations of the present disclosure, a nanoparticle may be substantially free of a distinct crosslinking agent and may be self-cross-linked. In such variations, the protein may be a relatively large water-soluble protein, for example, having an average

molecular weight of greater than or equal to about 8 kDa to less than or equal to about 700 kDa. Further, the water-soluble proteins comprise disulfide bonds in their sequence to facilitate self-cross-linking. The cross-linked water-soluble protein may be selected from the group consisting of: albumin, ovalbumin, human serum albumin, bovine serum albumin, transferrin, hemoglobin, IgG, enzymes, transport proteins, storage proteins, antibodies, aptamers, chemokines, hormonal proteins, polypeptides, and combinations thereof. In certain variations, the self-cross-linked water-soluble protein defines a mesh structure, such as those described above.

[0102] Any of the nanoparticles described above may also comprise one or more additional active ingredients, imaging agents, or targeting moieties, by way of example, as will be discussed further herein. As noted above, these compounds or moieties may be disposed in the openings of a mesh structure defined by the cross-linked protein and optionally the crosslinking agent reacted thereto. In certain aspects, the protein-based nanoparticles comprise at least one therapeutic or pharmaceutically active ingredients, such as exclusive or generic pharmaceutical active ingredients/drugs, new chemical entities, and combinations thereof. In accordance with certain aspects of the present disclosure, the nanoparticles are suitable for use in a wide variety of biofunctional or bioactive applications. A “biofunctional” or “bioactive” substance refers to a chemical substance, such as a small molecule, macromolecule, metal ion, or the like, that causes an observable change in the structure, function, optical function, or composition of a cell when a cell is exposed to such a substance. Examples of observable changes include increased or decreased expression of one or more mRNAs, increased or decreased expression of one or more proteins, phosphorylation of a protein or other cell component, inhibition or activation of an enzyme, inhibition or activation of binding between members of a binding pair, an increased or decreased rate of synthesis of a metabolite, increased or decreased cell proliferation, changes in optical properties, and the like. In certain aspects, the nanoparticles of the disclosure deliver active ingredients to a target, in some embodiments, to tissue or an organ of an organism. In other aspects, the nanoparticles provide binding to certain target regions in an organism to modify optical or physical properties to improve diagnostic procedures. Various categories of active ingredients are discussed herein; however, it will be understood that while general attributes of each of the categories of components may differ, there may be some common attributes and any given material may serve multiple purposes within two or more of such listed classes or categories.

[0103] In various aspects, the protein-based nanoparticles according to certain aspects of the present disclosure fulfill one or more of the following advantages. First, the protein-based nanoparticles provide the ability to design drug delivery vehicles for various active ingredient types and concentrations. Second, the protein-based nanoparticles circulate and remain for long periods within the organism, thus avoiding filtration and removal, immune system recognition

and/or complement activation. Third, the protein-based nanoparticles can provide an active targeting ability to deliver highly specific active ingredients to target tissues (for example, to a tumor site) to minimize systemic effects. This is particularly advantageous for chemotherapeutic treatments for cancer, where damage of attendant tissues can be minimized. Lastly, the protein-based nanoparticles can provide functional imaging that allows for distinguishing specific and non-specific binding.

[0104] The protein-based nanoparticles may comprise an active ingredient. An active ingredient is a compound or composition that diagnoses, prevents, or treats a physiological or psychological disorder or condition, or can provide a cosmetic or aesthetic benefit. In certain aspects, an active ingredient agent is targeted to a particular target, such as organs, tissues, medical implants or devices, hair, skin, mouth, eyes, circulatory system, and the like. For example, in various aspects, the nanoparticles having one or more active ingredients can be used in various pharmaceutical and/or cosmetic compositions. A “pharmaceutically and/or cosmetically acceptable composition” refers to a material or combination of materials that are used with mammals or other organisms having acceptable toxicological properties for beneficial use with such an animal. Pharmaceutically and/or cosmetically acceptable compositions include drug and therapeutic compositions, oral care compositions, nutritional compositions, personal care compositions, cosmetic compositions, diagnostic compositions, and the like.

[0105] In various aspects, the protein-based nanoparticles may be used in a wide variety of different types of compositions having a bio-functional or bioactive material and are not limited to the variations described herein. However, the present disclosure contemplates nanoparticles comprising one or more active ingredients that provides a diagnostic, therapeutic, prophylactic, cosmetic, sensory, and/or aesthetic benefit to an organism, such as a mammal. In certain aspects, an active ingredient prevents or treats a disease, disorder, or condition of hard or soft tissue in an organism, such as a mammal. As a non-limiting example, the current technology provides a method of treating a subject having cancer, the method comprising administering to the subject the protein-based nanoparticles in an effective amount to treat the cancer, wherein the cancer can be, for example, a carcinoma, a sarcoma, leukemia, a lymphoma, a myeloma, a germinoma, or a brain or spinal cord cancer (*e.g.*, glioblastoma). The cancer can be glioblastoma, brain cancer, breast cancer, stomach cancer, colon cancer, rectal cancer, liver cancer, pancreatic cancer, lung cancer, cervical cancer, uterine cancer, ovarian cancer, prostate cancer, testicular cancer, bladder cancer, renal cancer, head and neck cancer, throat cancer, Hodgkin's disease, non-Hodgkin's lymphoma, multiple myeloma, leukemia, or skin cancer.

[0106] The ensuing description of suitable active ingredients is merely exemplary and should not be considered as limiting as to the scope of active ingredients that can be introduced into the protein-based nanoparticles according to the present disclosure, as all suitable active

ingredients known to those of skill in the art for these various types of compositions are contemplated. Suitable active ingredients for use in such pharmaceutically and/or cosmetically acceptable compositions are well known to those of skill in the art and include, by way of example, pharmaceutical active ingredients found in the Merck Index, An Encyclopedia of Chemicals, Drugs, and Biologicals, Thirteenth Edition (2001) by Merck Research Laboratories and the International Cosmetic Ingredient Dictionary and Handbook, Tenth Ed., 2004 by Cosmetic Toiletry and Fragrance Association, each incorporated herein by reference. Each additional reference cited or described herein is hereby expressly incorporated by reference in its respective entirety. Certain suitable active ingredients, or pharmaceutically active ingredients or drugs, are known to those of skill in the art and include, but are not limited to, low-molecular weight molecules, quantum dots, natural and artificial macromolecules, such as proteins, sugars, peptides, DNA, RNA (including short interfering RNA (siRNA), *e.g.*, siRNA against STAT3).

[0107] A variety of low molecular weight molecules can be employed, particularly those having a molecular weight of less than about 10,000, optionally less than about 1,000, and optionally less than about 500. Suitable therapeutic active ingredients may include anti-proliferative agents; anti-rejection drugs; anti-thrombotic agents; anti-coagulants; antioxidants; free radical scavengers; nutrients; nucleic acids; saccharides; sugars; nutrients; hormones; cytotoxin; hormonal agonists; hormonal antagonists; inhibitors of hormone biosynthesis and processing; antigestagens; antiandrogens; anti-inflammatory agents; non-steroidal anti-inflammatory agents (NSAIDs); antimicrobial agents; antiviral agents; antifungal agents; antibiotics; chemotherapy agents; antineoplastic/ anti-miotic agents; anesthetic, analgesic or pain-killing agents; antipyretic agents, prostaglandin inhibitors; platelet inhibitors; DNA de-methylating agents; cholesterol-lowering agents; vasodilating agents; endogenous vasoactive interference agents; angiogenic substances; cardiac failure active ingredients; targeting toxin agents; vitamins; nutraceuticals; and combinations thereof. The description of these suitable organic compounds/pharmaceutical active ingredients/new chemical entities is merely exemplary and should not be considered as limiting as to the scope of compounds or active ingredients which can be applied to a surface according to the present disclosure, as all suitable organic molecules and/or active ingredients known to those of skill in the art for these various types of compositions are contemplated. Furthermore, an organic compound may have various functionalities and thus, can be listed in an exemplary class above; however, may be categorized in several different classes of active ingredients.

[0108] By way of example, suitable active ingredient molecules include chemotherapeutic drugs, such as doxorubicin (molecular mass of about 543.5 g/mol); paclitaxel or Taxol™ (molecular mass of about 853.9 g/mol), cholesterol lowering drug, lovastatin (molecular mass of about 404.5 g/mol), NSAID analgesic ibuprofen (molecular mass of 206.3 g/mol). Quantum dots

are optically active nanostructures, for example, cadmium tellurium (CdTe). Macromolecules include a wide range of compounds, generally including polymers and biomolecules having relatively large molecular weights. Such macromolecules can be naturally occurring or synthesized. Certain amino acids, peptides (amino acids linked via peptide bonds); polypeptides (linear chains of peptides), and even other and proteins (primary, secondary, and tertiary folded polypeptides), including enzymes are contemplated as active ingredients. Exemplary active ingredient proteins include heat shock protein 70 (HSP70) for dendritic cells and folic acid for cancer cells. Exemplary toxins for use as active ingredients include saporin and Botulinum toxins. Exemplary sugars include silylic acid leucocytes and glucuronic acid, for example. In some aspects, the therapeutic active ingredient is a nanoparticle or nanocrystal having a particle size that is below the particle size of the protein-based nanoparticles. For example, the nanoparticles or nanocrystals may have a particle size of less than about 50 nm, optionally less than about 20 nm, and in some aspects, less than 10 nm. Useful active ingredient nanoparticles include magnetite (Fe₃O₄), magnesium oxide, and metal based nanoparticles, comprising gold, silver, and the like. In other embodiments, the protein-based nanoparticle does not include other nanoparticles (*e.g.*, the protein-based nanoparticle may be substantially free of other nanoparticles (*e.g.*, metal-based nanoparticles (*e.g.*, metal oxide nanoparticles))).

[0109] In certain variations, the nanoparticle may comprise a therapeutic active ingredient is selected from the group consisting of: a drug, a steroid, and combinations thereof. In certain variations, the drug is selected from the group consisting of: paclitaxel, cis-platin, doxorubicin, and combinations thereof. In other variations, the therapeutic active ingredient is selected from the group consisting of a nucleic acid (*e.g.*, DNA, RNA (*e.g.*, mRNA, tRNA, rRNA, snRNA, or siRNA, *e.g.*, siRNA against STAT3), a plasmid, CRISPR CAS-9, or an aptamer), a protein or peptide (*e.g.*, an antibody or other targeting molecule), a chemokine, a peptide drug, and combinations thereof.

[0110] In other variations, the active ingredient of the nanoparticles of the disclosure may be used for diagnostic purposes, and may be considered to be an imaging agent, such as in various diagnostic medical imaging procedures (for example, radiographic imaging (X-ray), fluorescence spectroscopy, Forster/fluorescent resonance energy-transfer (FRET), computed tomography (CT scan), magnetic resonance imaging (MRI), positron emission tomography (PET), other nuclear imaging, and the like). Active imaging agents for use with diagnostic imaging include contrast agents, such as barium sulfate for use with MRI, for example or for example fluorescein isothiocyanate (FITC).

[0111] In other aspects, the protein-based nanoparticles may include other ingredients like polymers, dyes and colorants, inorganic ingredients including nanoparticles, nanomaterials, and nanocrystals, fragrances, and mixtures thereof.

[0112] In certain aspects, the protein-based nanoparticles can be provided in pharmaceutical compositions. In certain pharmaceutical compositions, the active ingredient is provided in a suitable pharmaceutical excipient, as are well known in the art. Thus, administration of protein-based nanoparticles in a pharmaceutical composition can be, for example, intravenous, topical, subcutaneous, transcutaneous, intramuscular, oral, intra-joint, perenteral, peritoneal, intranasal, by inhalation, or within or coating a medical device or implant. Pharmaceutical compositions are optionally provided in the form of solid, semi-solid, lyophilized powder, or liquid dosage forms, such as, for example, tablets, pills, capsules, powders, solutions, suspensions, emulsions, suppositories, retention enemas, creams, ointments, lotions, aerosols or the like, in unit dosage forms suitable for administration of precise dosages.

[0113] In accordance with certain aspects of the present disclosure, advanced design of protein-based nanoparticles can make them capable of promoting active ingredient delivery to a localized region, such as cancer targeting. In certain aspects, the nanoparticles can serve as targeting elements for circulating blood cells carrying the active ingredient payload (*e.g.*, chemotherapy drug) to a tumor. In certain embodiments, the protein-based nanoparticles can comprise a targeting moiety. For example, a targeting moiety, such as an antibody, a peptide, a ligand, or an aptamer, a liposome, a polysome, micelles, dendrimers, surface active agents (*e.g.*, PEG-ylated or having a surface bearing a zwitterion), and the like, which may be conjugated with an active ingredient itself or with the nanoparticles loaded with an active therapeutic agent. A nanoparticle can be designed to have such properties by providing such materials within the material forming the protein-based nanoparticle, or may be provided by later treating, reacting, or coating the material forming the protein-based nanoparticle to achieve such properties.

[0114] In various aspects, the protein-based nanoparticles can deliver an effective amount of the active ingredient to a target region within an organism. An “effective” amount of an active ingredient is an amount that has a detectable effect for its intended purpose and/or benefit. Preferably, the effective amount is sufficient to have the desired therapeutic, nutritional, cleansing, aesthetic, diagnostic, and/or prophylactic effect on the target region of an organism (*e.g.*, a mammal) to whom and/or to which the composition comprising the protein-based nanoparticles is administered. The specific effective amount of the active ingredient, including appropriate dosages and concentrations, will vary with such factors as the composition in which the active ingredient is provided, the site of intended delivery, the route of administration, the particular condition or subject being treated, the nature of concurrent therapy (if any), the specific active used, the specific dosage form, and the carrier employed, all of which are well known to those of skill in the art.

[0115] In certain aspects, a safe and effective amount of an active ingredient in a protein-based nanoparticle is about 0.001 to about 75 weight % of the total weight of phase (on a dry

basis). It should be noted that where the nanoparticle is distributed in a carrier or composition, that the overall concentration will be significantly less than in the nanoparticle particle themselves. In other aspects, the active ingredient is present in the protein-based nanoparticles at from about 0.01 to about 50%; optionally of about 1% to about 40%; optionally of about 1% to about 20%; and optionally 5% to about 20%. However, as discussed above, the concentration of active ingredient is highly dependent on various factors well known to those of skill in the art, including required dosage for the target region, bioavailability of the active ingredient and the release kinetics of the protein-based nanoparticles in which the active ingredient is located, among others. By using electrohydrodynamic jetting to form the nanoparticles, as will be described in greater detail herein, a high loading of drug into the protein carrier is possible. Protein-based nanoparticles with active ingredient loadings above 50% are possible.

[0116] In yet other variations, a nanoparticle comprising a cross-linked water-soluble protein having an average molecular weight of greater than or equal to about 8 kDa and less than or equal to about 700 kDa and may comprise a biomolecule, such as DNA, RNA, plasmids, siRNA, mRNA, transfer RNA, ribosomal RNA, small nuclear RNA, single stranded DNA, CRISPR CAS-9, aptamers, antibodies, peptides, targeting molecules, vitamins, and any combinations thereof.

[0117] In certain other aspects, the present disclosure contemplates a method of making a nanoparticle. The method includes jetting a liquid through a nozzle. The liquid comprises a water-soluble protein having an average molecular weight of greater than or equal to about 8 kDa and less than or equal to about 700 kDa and water (*e.g.*, water containing one or more solutes, *e.g.*, buffers). The method also includes exposing the liquid to an electric field sufficient to solidify the liquid and form the nanoparticle defining a mesh structure having an average linear mesh size of greater than or equal to about 1 nm to less than or equal to about 4 nm. Thus, the methods provided herein may be considered to be electrified jetting, such as that disclosed by Roh et al. in “Biphasic Janus Particles With Nanoscale Anisotropy”, Nature Materials, Vol. 4, pp. 759-763 (October, 2005), as well as in U.S. Patent No. 7,767,017 to Lahann et al. The contents of each of these respective references are hereby incorporated by reference in their respective entireties. However, it should be noted that the techniques described in the Roh and Lahann et al. references pertain to polymers rather than proteins, as described herein.

[0118] Electrified jetting is a process used to develop liquid jets having a nanometer-sized diameter, using electro-hydrodynamic forces. As shown in FIG. 3, an electrohydrodynamic jetting system 100 includes a source 110 of a liquid 112 contained in a channel 114 that is fed to a nozzle 120. A syringe pump (not shown) may be used to drive the liquids 112 into the nozzle 120. At the nozzle 120, a pendant droplet 132 is formed of conducting liquid 112. The nozzle 120 is in electrical communication with a power supply 122 that can be applied during the jetting operation. As shown,

there is also an electrically conductive and grounded plate 124 disposed below and spaced apart from the nozzle 120. The power supply 122 is also in electrical communication with the plate 124. Thus, the droplet 130 is exposed to an electric potential of a few kilovolts generated by the power supply 122, where the force balance between electric field and surface tension causes the meniscus of the pendent droplet 130 to develop a conical shape, the so-called Taylor cone (not shown). Above a critical point, a highly charged liquid jet or ejected stream 132 is ejected from an apex of the cone.

[0119] In one variation, the electric field is generated by the potential difference between nozzle 120 and plate 124. Typically, an electric field is formed by applying a potential difference between at least two electrodes from about 0.1 kV to about 25 kV (*e.g.*, from about 0.1 kV to about 0.5 kV, from about 0.5 kV to about 1.0 kV, from about 1.0 kV to about 5 kV, from about 5 kV to about 10 kV, from about 10 kV to about 15 kV, from about 15 kV to about 20 kV, or from about 20 kV to about 25 kV, *e.g.*, about 0.1 kV, about 0.5 kV, about 1.0 kV, about 2.0 kV, about 5.0 kV, about 10 kV, about 15 kV, about 20 kV, or about 25 kV). Various configurations of plates and geometries may be used to generate the electric field as known to those of skill in the art and are contemplated by the present disclosure. In the variation shown in FIG. 3, the ejected stream 132 is fragmented due to instabilities generated by the electric field, thereby forming a spray of droplets 140 that form the protein-based nanoparticles 142. The solvents in the liquid 112 in the ejected stream 132 are rapidly removed (*e.g.*, volatilized or evaporated) from the stream during the jetting process.

[0120] Morphological control can be achieved with the exemplary electric jetting formation methods described herein. Therefore, the ejected stream 132 exiting the nozzle 120 as a pendant droplet 130 can be fragmented to small droplets 140, instead of sustained and elongated jetting that leads to a continuous fiber. The size of the droplets 140 can also be controlled. Such control is attained by changing either the material properties of jetting liquids or the working parameters of electrified jetting that breaks-up the jet stream. It should be appreciated, however, that the final morphology of the ejected stream 132 is not always the same as those of the solid nanoparticle 142 products collected on the substrates. The shape of final nanoparticles 142 can also be controlled by a sol-gel transition process or by subsequent processing after formation by electric jetting.

[0121] Since the electrified jetting methods are related to electrohydrodynamic processes, the properties of the jetting liquid and operating parameters are interrelated. Moreover, when the jetting liquids are not one-component systems (*i.e.*, mixtures of two or more compounds), the jetting liquid is a solution having properties governed by several parameters of the solvent and solutes. It should be appreciated that liquid properties, solution parameters, and operating parameters are related, as recognized by those of skill in the art. Relevant material properties include viscosity, surface tension, volatility, thermal and electrical conductivity, dielectric

permittivity, and density. Relevant solution properties include concentrations, molecular weight, solvent mixtures, surfactants, doping agent, and crosslinking agents. Finally, relevant operating parameters include flow rate of the liquid streams, electric potential, temperature, humidity, and ambient pressure. With regard to the operating parameters, the average size and size distributions of the droplets in electrospraying with cone-jet mode also are believed to be dependent on the flow rate (*e.g.*, pumping rate of the jetting liquid). At a fixed flow rate, one or several relatively monodisperse classes of nanocomponent diameters are formed. At minimum flow rate, the modality of the distributions and diameter of the droplet itself also show their minima. When the flow rate is changed, the electric field can be adjusted by changing either distance or electric potential between the electrodes in order to sustain a stable cone-jet mode. Higher flow rates may be accompanied by a higher electrical field applied for mass balance of jetting liquids. When the diameter of droplets is larger than desired, solvent evaporation does not fully occur before the droplets reach the collecting substrate, so the resulting droplets may be wet and flat.

[0122] In various aspects, the use of the electric jetting methods provided herein afford greater control over the morphology and design of the protein-based nanoparticles as opposed to other methods of forming nano-components (such as precipitation, sonication during liquid jetting, and the like). One of the major differences between electrohydrodynamic co-jetting of polymers, as had been done in the past, and protein nanoparticles is the fact that the components react with each other during jetting. For protein jetting, the initial solution was expected to be unstable and it was surprising and unexpected that polymer-containing solutions could be jetted.

[0123] In certain aspects, the liquid comprises water and optionally aqueous carriers. The liquid may include components that adjust pH, such as acids (*e.g.*, acetic acid) or bases. In certain variations, the liquid may comprise a crosslinking agent. During the exposing, the water-soluble protein is at least partially cross-linked. However, the cross-linking may continue after the nanoparticle is formed and solidifies.

[0124] As noted above, the nanoparticle formed by such a process may comprise a cross-linked water-soluble protein that defines a mesh structure having an average linear mesh size of greater than or equal to about 1 nm to less than or equal to about 4 nm. In certain variations, the liquid further comprises an additive selected from the group consisting of: a therapeutic active ingredient, an imaging agent, a biomolecule, a targeting moiety, and combinations thereof. The additive is thus incorporated into the nanoparticle.

[0125] It should be noted that electrohydrodynamic jetting methods of proteins form different products from other techniques used to form protein particles. In the electrohydrodynamic jetting method, the droplets produced are sufficiently small that complete solvent evaporation over millisecond characteristic times does not require pre-heating of the solution or bath. The solid

protein nanoparticles are collected on the grounded plate (*e.g.*, plate 124). Consequently, there is no risk of thermally damaging dissolved labile molecules and the protein itself.

[0126] In contrast, there are different conventional methods to fabricate protein nanoparticles. In desolvation/coacervation, the addition of a “desolving” agent, such as ethanol or acetone, is required. The desolving agent then dehydrates the protein resulting in conformational change from a stretched to coil conformation. The proteins can be cross-linked, commonly by using glutaraldehyde, but the protein has undergone a conformational change that can be undesirable.

[0127] Protein nanoparticles can also be stabilized by denaturing the protein before desolvation, for example, by using a reducing agent such as BME (β -mercaptoethanol). In other techniques, emulsification is used by mixing an aqueous protein solution with an immiscible oil or organic solvent solution, and sonicating the mixture. This causes the proteins to homogenize and form small droplets. The solvent/nonsolvent is subsequently removed to yield nanoparticles. However, the sonication process can damage the native protein structure.

[0128] In yet other variations, thermal gelation is used by stirring proteins while they are being denatured at a high heat. The proteins unfold, aggregate, and then the growth of nanoclusters is stopped by immediately sinking them in an ice water bath. They can restabilize through disulfide bonds and hydrogen bonding.

[0129] In these methods, protein nanoparticle fabrication requires addition of alcohols, as moderately hydrophobic solvents, that destabilize the protein native structures, or they require a reducing agent such as BME that denatures the protein. In the emulsification, high sonication is required that can damage protein native structure. In the thermal gelation, the protein is denatured at a high heat.

[0130] However, by using the present electrohydrodynamic jetting methods, the protein-based nanoparticles may be substantially free of denaturing or conformational changes in the protein, aside from crosslinking. CD spectroscopy conducted on albumin-based nanoparticles prepared by such a technique show that albumin is not denatured in human serum albumin nanoparticles. Also in the case of ovalbumin nanoparticles, the particles still maintain their bioactivity after electrohydrodynamic jetting, which successfully results in activation of immune cells.

[0131] In certain aspects, a protein-based nanoparticle comprises a plurality of proteins. A majority of proteins maintain a predominantly native conformation. Proteins with a native conformation show similar or identical circular dichroism spectra to the proteins in the free state. In certain aspects, the majority of proteins that maintain a predominantly native conformation is greater than or equal to about 75% of the proteins in the nanoparticle, optionally greater than or equal to about 80% of proteins in the nanoparticle, optionally greater than or equal to about 85% of proteins in the nanoparticle, optionally greater than or equal to about 90% of proteins in the

nanoparticle, optionally greater than or equal to about 95% of proteins in the nanoparticle, and in certain aspects, optionally greater than or equal to about 97% of proteins in the nanoparticle maintain a native confirmation of the free state of the protein.

[0132] In certain other variations of the present disclosure, as shown in FIG. 4, an electrohydrodynamic jetting system 200 is used to form a multicompartmental protein-based nanoparticle. The design of the electrohydrodynamic jetting system 200 is modified from that shown in the electrohydrodynamic jetting system 100, because it involves a side-by-side electrojetting apparatus that provides at least two distinct liquid streams that are co-jetted together to form a multicompartmental nanoparticle. In order to incorporate two different liquid streams, a first source 210 of a first liquid 212 feeds a first channel 214 that is fed to a nozzle 216. A second source 218 of a second liquid 220 feeds second channels 222 that also feed into nozzle 216. The first and second channels 214, 222 are configured adjacent to each other (*i.e.*, side by side) in nozzle 216. In some variations, first and second channels 214, 222 are capillaries. Thus, the first and second channels 214, 222 feed two different jetting liquid streams 212, 220 into a region 226 having an electric field generated by a power supply 230. First and second channels 214, 222 are of sufficient dimensions to allow contacting of liquids streams 212, 214 to form composite stream 232. In one variation, this electric field is generated by the potential difference between nozzle 216 and the electrically conductive and grounded plate 234. Like the electric field described above in the context of FIG. 3, the electric field is formed by applying a potential difference between the at least two electrodes from about 0.1 kV to about 25 kV. Various configurations of plates and geometries may be used to generate the electric field as known to those of skill in the art and are contemplated by the present disclosure.

[0133] A droplet 240 is exposed to an electric potential of a few kilovolts generated by the power supply 230 in the region 226, where the force balance between electric field and surface tension causes the meniscus of the pendent droplet 240 to develop a conical shape, the so-called Taylor cone (not shown). Like the process described above, at a critical point, a highly charged liquid jet or ejected composite stream 232 is ejected from an apex of the cone.

[0134] The ejected stream 232 is fragmented due to instabilities generated by the electric field, thereby forming a spray of droplets 244 that form the protein-based nanoparticles 246. The solvents in the first and second liquids 212 and 220 in the ejected stream 232 are rapidly removed (*e.g.*, volatilized or evaporated) from the stream during the jetting process. In this manner, a plurality of multicompartmental protein-based nanoparticles 248 are formed. Each protein-based nanoparticle 248 includes a first compartment (*see e.g.*, “A” in the precursor droplet 240) defining at least a portion of an exposed surface of the multicompartmental nanoparticle 248. The nanoparticle 248 also includes at least one additional compartment (*see e.g.*, “B” in the precursor

droplet 240) constituting (*e.g.*, defining) at least a portion of an exposed surface and comprising at least one additional composition distinct from the first composition. By a distinct composition, it is meant at least one composition comprises a distinct chemical component or in certain alternative variations, the compositions may have the same constituents, but have differing amounts of the constituents. In certain variations, the first compartment may have the first composition comprising a water-soluble polymer having an average molecular weight of greater than or equal to about 8 kDa and less than or equal to about 700 kDa.

[0135] In certain variations, the first compartment may further comprise a crosslinking agent reacted with the water-soluble protein in the first compartment. The first composition may be any of those described above previously in the context of the single compartment nanoparticle having the cross-linked water-soluble protein. The one or more additional compartments may also have a protein and an optional crosslinking agent.

[0136] In one variation, the first composition in the first compartment comprises a water-soluble protein is selected from the group consisting of: ovalbumin, albumin, mucin, transferrin, insulin, lysozyme, hemoglobin, collagen, and combinations thereof. In certain variations, the at least one additional compartment also comprises a second water-soluble protein. The water-soluble protein may be provided in an aqueous liquid comprising water during the jetting process. The second water-soluble protein may be selected from the group consisting of: ovalbumin, albumin, mucin, transferrin, insulin, lysozyme, hemoglobin, collagen, and combinations thereof.

[0137] Each respective compartment can incorporate different: (i) active ingredients or drugs; (ii) different mesh sizes, so the release profile from each compartment can differ; and/or (iii) each crosslinking method can be different so the release of the active ingredient/cargo can be tuned. Further, one of the compartments can serve as an imaging modality (thus comprising an imaging agent), while the other serves as a drug carrier (thus comprising a therapeutic active ingredient). Further, in the case of antigen delivery, each compartment can be made from a different antigen, so multi-antigen delivery is possible. Furthermore, the surface of select compartments can be modified with different agents, stealth or cloaking moieties, or targeting moieties.

[0138] Various embodiments of the inventive technology can be further understood by the specific examples contained herein. Specific Examples are provided for illustrative purposes of how to make and use the compositions, devices, and methods according to the present teachings and, unless explicitly stated otherwise, are not intended to necessarily be limiting or a representation that given embodiments have, or have not, been made or tested.

[0139] **Example 1**

[0140] Protein Nanoparticle Fabrication – Antigen-Based Nanoparticles

[0141] Ovalbumin (OVA) protein nanoparticles were prepared using electrohydrodynamic (EHD) jetting, as described above. Ovalbumin (Sigma Aldrich, USA) was dissolved in endotoxin-free water (G-Biosciences, USA) and run through endotoxin removal spin columns (ThermoFisher Scientific) according to manufacturer's instructions to provide endotoxin-free OVA. Then, OVA protein at 7.5 w/v% (g/ml) and poly(ethylene glycol)-based crosslinking agent (with molecular weight of 2 kDa NHS-PEG-NHS) with desired ratios (5, 10, 30 or 50 w/w protein %) are prepared in solvent mixture of water and ethylene glycol with ratios of 80:20 vol. % or 40:60 vol. % depending on the formulation. Then, the solution was mixed on the shaker until the crosslinker was fully dissolved. Afterwards, a syringe was filled with dissolved protein and crosslinking agent in water-ethylene glycol mixture. The syringe was capped with 26G needle, which was used as a capillary and placed into the pump. The solution was pumped at 0.1 mL/h. Upon formation of a droplet at the tip of the needle, the electric field was applied to the system. After applying approximately 10–12 kV of voltage, the droplet was distorted to a stable cone, Taylor cone, and formed a protein jet. The protein jet then split into individual droplets with higher surface area. This induced instantaneous evaporation of the solvent and resulted in solidification of the non-volatile components. Therefore, polymerized OVA nanoparticles were sprayed to the grounded collecting plate. The distance between the needle tip and the collector sheet was adjusted to 15 cm–20 cm.

[0142] By using homo-bifunctional polyethylene glycol crosslinker, O'-bis[2-(N-Succinimidyl-succinylamino)ethyl] polyethylene glycol (NHS-PEG-NHS) (Sigma Aldrich, USA) as a crosslinker, the PEG units formed amide bonds with amino groups in OVA such as lysine residues. To ensure completion of crosslinking reaction, OVA nanoparticles were kept at 37 °C for 7 days. This resulted in formation of stable, polymerized OVA nanoparticles. After stable nanoparticles were formed in solid phase, nanoparticles were collected in Endotoxin-Free Dulbecco's PBS (EMD Millipore, USA.) containing 0.01% tween 20.

[0143] Parameters, such as protein concentration, solvent viscosity and solvent dielectric constant, can be adjusted to control the size and network structure in pNPs. To increase the size of hydrated OVA pNPs to 500 nm, the ratio of water-to-ethylene glycol was decreased to 40:60 (vol. %), which effectively decreased the overall dielectric constant of the solvent system and increased nanoparticle size. However, additional optimization was required to obtain 500 nm OVA pNPs. First, the PEG/OVA ratio was decreased to 5% (w/w). Second, the molecular weight of the PEG crosslinker was increased from 2 kDa to 20 kDa. Through these modifications, hydrated OVA pNPs with a size of 500 nm were reliably prepared, as confirmed by dynamic light scattering.

[0144] Using protein-based nanoparticles (pNPs) comprised of the actual antigen eliminates the need for a separate nanoparticle carrier. If the entire particle, or a majority of the particle, is comprised of antigen, pNPs have the potential for enhancing DC surface receptor engagement, prolonging tissue persistence, sustaining antigen activity and minimizing off-target material delivery. In the past, proteins have been assembled into particles through structurally ordered assembly, unstructured hydrophobic assembly and electrostatic assembly. However, the protein design must achieve self-assembly with desired materials physicochemical properties and desired immune interactions. Protein assembly through fusion and sequence modification are more affected by antigenic variability in their ability to self-assemble and preserve antigen recognition. Compared to fusion and sequence modification, chemical conjugation to other proteins, lipids or polymers, promises versatility and broader applicability to a wider spectrum of antigens, but requires multiple processing steps. For example, cross-linked peptide nanoclusters are fabricated for delivery of oncofetal antigen by desolvation and are stabilized through disulfide bonds. However, changes to the primary structure of the protein, such as addition of cysteine to the c-terminus of the peptide, is necessary to ensure successful crosslinking.

[0145] The pNPs comprised of polymerized ovalbumin (OVA) linked by poly(ethylene glycol) (PEG) units provide the ability to reduce off-target immune responses, because the target antigen becomes the main structural building block of the pNPs. This novel type of pNPs ensures presentation of dense arrays of antigen readily recognizable by antigen-presenting cells (APCs). In pNPs comprised of polymerized OVA, antigen presentation is influenced by the crosslinker:protein ratio. Specifically, the four types of polymerized OVA pNPs are evaluated with various PEG:OVA ratios in terms of their uptake by dendritic cells, T cell activation, lymphatic drainage, antibody production, and anti-tumor efficacy.

[0146] Characterization of PNPs

[0147] Scanning electron microscopy (SEM). SEM images were recorded using a FEI Nova 200 Nanolab SEM/FIB at the Michigan Center for Materials Engineering at acceleration voltages of 5kV. Images were processed using ImageJ (Wayne Rasband, NIH) to obtain the respective nanoparticle size distribution. For particle size determination, >500 particles/sample were measured using ImageJ.

[0148] Dynamic/electrophoretic light scattering (DLS/ELS). DLS/ELS measurements were carried out using a Zetasizer Nano ZS (Malvern Panalytical). DLS was employed to measure the particle size distribution in PBS buffer after particle collection. ELS was employed to determine the zeta potential of OVA NPs. 3 individual measurements were carried out per sample and averaged to determine the particle size and zeta potential.

[0149] Atomic force microscopy (AFM). AFM measurements were carried out using an MFP-3D (Oxford Instruments, UK) using CSC-38noAl-A cantilevers (Micromash, USA) with a spring constant of 0.09 N/m. Samples were prepared by electrospraying OVA pNPs directly onto silicon substrates coated with poly(4-Penta fluorocphenyl-p-xylylene) via chemical vapor deposition (CVD) polymerization (see supporting information); the substrates were allowed to crosslink at 37 °C for several days prior to use. OVA NPs were localized by scanning the surface in tapping mode over a (5 x 5) μm^2 area and then decreasing the scan area for visualization of a single NP. The force curves were obtained by indenting the tip into the center of an individual nanoparticle and recording the deflection of the cantilever.

[0150] Small angle neutron scattering (SANS). SANS experiments were carried out at the NIST Center for Neutron Research using the NGB 30 instrument. Using neutron wavelength of $\lambda = 6 \text{ \AA}$ and $\Delta\lambda/\lambda=0.11$ at detector distances 1.3 m, 4.0 m, and 13.2 m, we provided a q-range of 0.003 $\text{\AA}^{-1}$ to 0.5 $\text{\AA}^{-1}$. OVA pNPs with PEG/OVA ratio of 10% and 40% dispersed in D₂O (2 mg/mL) were loaded into 1 mm titanium scattering cells between mounted quartz windows, and a Julabo temperature-controlled bath was used to maintain the sample temperature at 37 °C. SANS data were then collected and reduced using the NCNR IGOR software. Data analysis was performed subsequently using the Sasview software.

[0151] Preparation of bone marrow-derived dendritic cells (BMDCs). BMDCs were prepared according to literature protocols. C57BL/6 mice were kept in a pathogen-free environment and allowed to acclimate for at least one week before experiments. Briefly, femur and tibia were harvested from C57BL/6 mice. Bone marrow was flushed with a syringe and collected. The cell suspension was passed through a 40 μm cell strainer. After centrifugation, cells were plated into non-tissue culture treated Petri-dishes at a concentration of 2 million cells per dish in DC media (RPMI 1640 supplemented with 10% FBS, 1% penicillin-streptomycin, 50 μM β -mercaptoethanol and 20 ng/ml GM-CSF) at 37 °C with 5% CO₂. The media was refreshed on days 3, 6, and 8. BMDCs were used for experiments on days 10–12.

[0152] OVA pNP uptake by BMDCs. Internalization of fluorescent OVA pNPs by BMDCs was visualized using confocal microscopy and quantified using flow cytometry. Fluorescent OVA pNPs were obtained by addition of AlexaFluor 647-conjugated albumin from bovine serum (BSA) at 1 mg/ml to the solvent mixture for electrospraying of the nanoparticles. For confocal imaging, BMDCs were seeded on chamber slides (105 cells/well) and maintained in a humidified incubator at 37°C and 5% CO₂. Cells were incubated with 10 $\mu\text{g/ml}$ of OVA NPs for 24 hours. The cells were then washed three times with PBS, fixed with 4% paraformaldehyde, washed, and permeabilized with 0.1% Triton-X solution that was followed by treatment with blocking solution of 1% BSA. The actin filaments were stained with AlexaFluor 488-Phalloidin

and the nucleus was stained with DAPI. The samples were imaged using a 63x oil-immersion lens on a Nikon A-1 spectral confocal microscope located at the Microscopy and Image Analysis Laboratory (MIL) at the University of Michigan.

[0153] For quantitative uptake studies, flow cytometry was carried out. BMDCs were plated in a 12-well plate at a density of 1 million cells per well in DC media. After 24 hours, media was removed from the wells to remove non-adherent cells, and fresh media containing different nanoparticle groups at 10 μ g/ml was added to the wells. After 24-hour incubation of cells with OVA nanoparticles, the cells were washed with PBS three times and then trypsinized. The cells were washed two more times and stained with DAPI before analyzing them via flow cytometry using a Cytoflex (Beckman Coulter) cell analyzer located at the Flow Cytometry Core of the University of Michigan. Data were analyzed using FlowJo software.

[0154] CFSE dilution assay. CFSE dilution assay was performed to evaluate the proliferation of OT-I CD8⁺ cells after co-culture with OVA pNP-treated BMDCs. BMDCs were seeded in 96-well plates at a density of 50,000 cells/well and then incubated with the respective OVA NPs groups, soluble OVA, SIINFEKL (positive control), and PBS (negative control) overnight. Naive CD8⁺ T cells were isolated from the spleen of OT-I transgenic mice using a magnetic CD8⁺ T-cell-negative selection kit. OT-I CD8⁺ cells were fluorescently labeled by incubation with CFSE (1 μ M) for 10 min at 37°C. CFSE-labeled OT-I CD8⁺ T cells were then co-cultured with OVA pNP-treated BMDCs in 96 well plates at a density of 50,000 cells/well for 72 hours. BMDCs were washed with PBS three times before co-culture. Cells were then stained with CD8 α -APC and DAPI, and flow cytometry (Cytoflex, Beckman Coulter) was used to determine the percentage of live, proliferated OT-I CD8⁺ cells. The data was processed using FlowJo software and reported as % CFSE dilution, which was proportional to OT-I CD8⁺ cell proliferation.

[0155] Immunization. Six-week-old, female C57BL/6 mice were purchased from Jackson Laboratory. Mice (n=5 per group) were immunized subcutaneously at the tail base at a dose of 10 μ g OVA with 15 μ g CpG in 100 μ l sterile PBS buffer (primary immunization). Boost immunization was performed on day 21 after primary immunization. On days 20 and 42, blood was collected by submandibular bleed for serum antibody titers analysis. To separate serum, the collected blood was centrifuged at 10,000 x g for 5 mins. Serum was then stored at -80°C until analysis.

[0156] For ELISA analysis, 96 well flat bottom Immunoplates (Thermo Scientific) were coated with 1 μ g/well OVA solution in 0.05 M carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. Plates were then washed with 50 mM Tris, 0.14 M NaCl, 10.05% Tween 20 (pH 8) followed by blocking with 50 mM Tris, 0.14 M NaCl, 1% BSA (pH 8) for 1 hour at room temperature. Samples were diluted in 50 mM Tris, 0.14 M NaCl, 0.05% Tween 20,

1% BSA and added to each well for an hour incubation at room temperature. After washing, the plates were incubated with diluted horseradish peroxidase enzyme (HRP) conjugated Rabbit anti-mouse IgG for an hour. The plates were then washed and incubated with TMB substrate solution for 10 minutes. The reaction was stopped by addition of 2 M H₂SO₄ solution. The plates were read at the wavelength of 450 nm using a plate reader.

[0157] Statistical analysis. All quantitative experiments were performed in triplicate and were presented as arithmetic mean $\pm$ SEM. Statistical analysis were performed using SPSS Statistics 24 software. One-way ANOVA with Tukey's post-test was used to determine significance among groups. A P-value of <0.05 was considered statistically significant (*P<0.05, **P<0.01, ***P<0.001; ****P<0.0001); P-values of >0.05 were considered not significant (ns).

[0158] FIGS. 5A-5D show SEM images of the different OVA pNPs as collected on the counter electrode. FIG. 5A shows an ovalbumin nanoparticle with 10 w/w% NHS-PEG-NHS crosslinker (MW=2000 g/mol (2k XL)), FIG. 5B shows 30 w/w% 2k XL, FIG. 5C shows 50 w/w% 2k XL fabricated with 80:20 vol. % ethylene glycol/water, and FIG. 5D shows 5 w/w% 20k XL fabricated with 40:60 vol. % ethylene glycol/water.

[0159] The pNPs were dispersed in PBS buffer, and their size was measured using DLS. The size of hydrated nanoparticles characteristically increased with lower PEG/OVA ratio (FIG. 6A). The swelling of the particles with respect to their SEM dry size was estimated using

$$\text{Swelling} = \frac{d_{\text{DLS}} - d_{\text{SEM}}}{d_{\text{SEM}}} \quad (\text{Equation 1})$$

where dDLS and dSEM are the nanoparticle diameters obtained from DLS and SEM. As shown in the table in FIG. 6B, swelling ratios of 1.9, 2.1, 1.4 and 1.1 were calculated for the OVA pNPs with PEG/OVA ratios of 5, 10, 30 and 50%, respectively. Thus, OVA pNPs with PEG/OVA ratios of 5% and 10% increased their size by almost 200% after storage in PBS, whereas PEG/OVA ratios of 50% resulted in about 100% swelling. These differences in the swelling behavior suggest substantial differences in the mesh sizes of the protein gels of which the pNPs are comprised. The dependency of pNP swelling on crosslinker amount indicates that preparing protein-based nanoparticles in accordance with the electrohydrodynamic jetting methods, in fact, does yield particles with different mesh size. However, the mesh size is also quantified more accurately using small-angle neutron scattering to evaluate the network density of polymerized pNPs.

[0160] Mesh size of OVA pNPs. SANS measurements of two representative OVA pNPs, 10% and 50% PEG/OVA NPs dispersed in D₂O (2 mg/mL) were taken. FIG. 6C shows SANS data and fits for the OVA pNPs with 10% and 50% XL. It was expected that the scattering from the hydrated protein network resembles the scattering from heterogeneous synthetic polymer hydrogels, which can be modeled as a disordered two-phase system with a protein-rich network

phase and a protein-poor polymer phase. Accordingly, the scattering curves were fitted to a combined Porod model and the Debye-Anderson-Brumberger (DAB) model (solid black lines in FIG. 6C) according to

$$I(q) = \frac{8\pi\Phi(1-\Phi)(\Delta\rho)^2\xi^3}{(1+(q\xi)^2)^2} + \frac{A}{q^4} \quad (\text{Equation 2})$$

5 where A is a coefficient that determines the relative magnitude of Porod scattering. The DAB model (first term in equation (2)) describes scattering from the concentration correlations between the protein-rich phase with volume fraction Φ and scattering length density contrast $\Delta\rho$ with the surrounding fluid that is randomly distributed into domains of average spacing, *i.e.*, mesh size (ξ). The Porod model (second term in equation (2)) represents scattering from smooth interfaces
10 between the protein-rich and protein-poor domains.

[0161] Equation (2) provides fits of the observed SANS spectra from the two samples. At low q-values, a q⁻⁴ dependence of the scattering data is observed, consistent with scattering from a smooth interface. At moderate q-values, the length scale, or mesh size (ξ), is apparent as a shoulder in the scattering curve. It can be noted that the overall fit for 50% PEG/OVA pNPs is
15 poor in the region where the Porod and the DAB model contributions are of similar magnitude (q~0.01-0.02 Å⁻¹). The explanation for this lies in the interference between the Porod scattering from the interfaces of the protein-rich domains and the DAB scattering from polymer chains inside the domains. This is not accounted for in the model and would likely show up in the mid q-range, where the model gives a poor fit.

20 [0162] The DAB scale factor ($8\pi\Phi(1-\Phi)(\Delta\rho)^2$) increases four-fold as the PEG/OVA ratio increased from 10% to 50%, confirming the densification of the protein network as the degree of crosslinking increases. Furthermore, the average spacing ξ decreases nearly two-fold as the PEG/OVA ratio increases from 10% to 50% (Table 1), thus revealing a more finely divided structure with increasing PEG/OVA ratio. Together, these results suggest that the protein network
25 becomes denser and more finely heterogeneous with increasing PEG/OVA ratio, consistent with a more porous but smaller mesh structure at higher crosslink density.

[0163] Table 1

	10 % PEG/OVA	50% PEG/OVA
Porod scale factor, A	$3.70 \times 10^{-9} \pm 2.99 \times 10^{-13}$	$5.92 \times 10^{-9} \pm 1.02 \times 10^{-13}$
DAB scale factor, $8\pi\Phi(1-\Phi)(\Delta\rho)^2$	$4.92 \times 10^{-6} \pm 1.32 \times 10^{-7}$	$31.95 \times 10^{-5} \pm 1.60 \times 10^{-7}$
Correlation length (mesh size (ξ)), (nm)	3.98 ± 0.12	2.15 ± 0.01

[0164] The uptake of polymerized OVA pNPs by BMDCs was evaluated quantitatively by flow cytometry (FIG. 7A) and further visualized by confocal microscopy (FIGS. 7B–7E). For this
30 purpose, OVA pNPs were fluorescently labeled by adding AlexaFluor 647-conjugated albumin

from bovine serum (BSA) to the OVA electrospraying solutions. The fluorescence intensity of OVA NPs (10 $\mu\text{g/ml}$) was quantified using a plate reader; no significant differences in fluorescence intensity between the different nanoparticle groups was found. Next, OVA pNPs were incubated with BMDCs for 24h at a concentration of 10 $\mu\text{g/ml}$. Cellular uptake was quantified using flow cytometry by comparing the mean fluorescence intensity (MFI) values. The data show that there is a difference in the MFI values for OVA pNPs with different crosslinking density. MFI values are increased for pNPs with lower PEG/OVA ratio (10%), which correlate with higher cellular uptake, compared to the other groups. Cells incubated with pNPs with a 10% PEG/OVA ratio showed 6.9-fold greater MFI than those exposed to pNPs comprised of 50% PEG/OVA ($P < 0.0001$). However, MFI values for cells incubated with pNPs with 5% and 10% PEG/OVA ratios were not statistically different ($P > 0.05$). It has been shown previously that the elasticity of nanoparticles affects cellular uptake: Nanoparticles with Young's moduli between 30 and 140 kPa show the highest uptake by RAW 264.7 macrophages, while softer (<30 kPa) or harder (>140 kPa) NPs show reduced uptake. Here, pNPs with PEG/OVA ratios of 5% and 10%, which had intermediate elasticity as indicated by E moduli of $E = 43$ kPa are associated with the highest levels of cellular uptake.

[0165] Antigen cross-presentation by OVA pNPs. Eliciting an effective immune response requires delivery of OVA to antigen-presenting cells (APCs), such as dendritic cells (DCs). DCs digest OVA through a process called cross-presentation, which results in the activation and proliferation of CD8⁺ T cells. Thus, the ability of OVA pNP-treated BMDCs to promote antigen cross-presentation and induce antigen-specific proliferation of OT-I CD8⁺ cells were evaluated using a CFSE dilution assay (FIGS. 3F and 3G). CFSE dilution was proportional to the proliferation of OT-I CD8⁺ cells. Therefore, BMDCs were first incubated with OVA pNPs or soluble OVA (control) at 10 $\mu\text{g/mL}$ for 24h. BMDCs were then co-cultured with CFSE-labeled naïve OT-I CD8⁺ T cells, which recognize the OVA-derived epitope SIINFEKL presented in the context of MHC-I H2Kb. After 72h of co-culture, the population of proliferated CD8⁺ T cells was assessed using flow cytometry. Proliferation was affected by the PEG/OVA ratios of the pNPs. The OVA pNPs with PEG/OVA ratio of 5% showed 4.4-fold ($P < 0.0001$) higher proliferation rates than pNPs with a 50% PEG/OVA ratio. Similarly, pNPs with PEG/OVA ratio of 10% showed 3.6-fold ($P < 0.001$) higher proliferation rates than pNPs comprised of 50% PEG/OVA. Cross-priming and proliferation of the OT-I CD8⁺ cells were significantly enhanced for OVA pNPs with 5% ($P < 0.0001$), 10% ($P < 0.0001$) and 30% ($P < 0.01$) PEG/OVA ratios as compared to solute OVA (Figure 3A). While all pNP groups outperformed solute OVA, 5% and 10% PEG/OVA pNPs were most efficient in promoting antigen cross-presentation and proliferation of OT-I CD8⁺ cells. This result suggests (1) greater uptake of 5% and 10% PEG/OVA pNPs by BMDCs and (2) facilitating the processing of OVA pNPs by BMDCs due to lower crosslinking

density and larger size of 5 and 10% PEG/OVA pNPs. There is some evidence that larger particles can direct antigen to the class I antigen presentation pathway more efficiently, which might explain the higher proliferation values for 5% PEG/OVA pNPs (500 nm vs. 200 nm). Once internalized by BMDCs, smaller particles are shuttled more rapidly to an acidic environment than larger ones, which can lead to fast and unregulated degradation and inefficient cross-presentation. Larger particles remain longer in a neutral environment, thus preserving the antigens for more efficient cross-presentation. The results here indicate that the PEG/OVA (protein to crosslinking agent) ratio is an important parameter for enhancing proliferation of CD8⁺ T cells, lower PEG/OVA ratios resulting in higher proliferation rates.

[0166] Humoral immune responses after subcutaneous delivery of OVA pNPs. In vivo performance of the pNPs was evaluated by evaluating their ability to induce humoral immune responses in mice. Following a prime-boost vaccine regimen shown in FIG. 8A, C57BL/6 mice were injected subcutaneously at the tail base with OVA pNPs of varying PEG/OVA ratio (10, 30, 50 %) and size (200 nm, 500 nm) or solute OVA (10 µg OVA/100 µL dose), co-administered with CpG (15 µg/dose). Boost immunization was performed on day 21 after primary immunization. Anti-OVA serum IgG responses were measured on days 20 and 42 using an ELISA assay. Compared to soluble OVA, pNPs with a 10% PEG/OVA ratio elicit 49.4-fold in anti-OVA serum IgG titers ($P < 0.0001$) and 9.1-fold increase in boost immunization ($P < 0.05$), respectively. These pNPs also outperformed pNPs comprised of 50% PEG/OVA, as indicated by a 1.9-fold increase in anti-OVA serum IgG titers after prime immunization ($P < 0.01$). The results show that 2 doses of OVA pNPs administered in a prime-boost regimen elicited stronger humoral immune responses than the equivalent doses and regimen of soluble OVA (FIGS. 8B–8C). While the larger, 5% PEG/OVA pNPs showed stronger CD8⁺ T cell responses in vitro, the same particles elicited a weaker humoral immune response in vivo (comparable to soluble OVA). Because the elasticity of 5 and 10% PEG/OVA pNPs is similar, the weaker humoral immune response of 5% PEG/OVA pNPs can be attributed to their larger size (500 nm). Larger pNPs may have limited lymphatic drainage due to extended tissue persistence at the injection site.

[0167] OVA pNPs delivery to lymph nodes. Next, the pNPs targeting of the draining lymph nodes using AlexaFluor 647-labeled pNPs was evaluated. OVA pNPs of varying PEG/OVA ratio (10, 30, 50 %) and size (200 nm, 500 nm) were injected subcutaneously at the tail base of C57BL/6 mice (10 µg OVA/100 µL dose). The inguinal draining lymph nodes were harvested 48 hours after injection. Single-cell suspensions from the draining lymph nodes were prepared and pNPs uptake among the different populations of antigen-presenting cells were analyzed (dendritic cells, macrophages and B cells) using flow cytometry by comparing the MFI values of the cells. It was found that the MFI values of F4/80⁺ macrophages, B220⁺ B cells and

CD11c+ DCs (FIGS. 8D–8F) increased with decreasing PEG/OVA ratio for the smaller (200 nm) pNPs with 10%, 30% and 50% PEG/OVA ratio. The MFI values of cells with larger (500 nm) 5% PEG/OVA pNPs were significantly lower, indicating that the pNPs were not delivered to draining lymph nodes efficiently due to their larger size. In the past, many different particle sizes have been studied with respect to their lymphatic drainage. It has been shown that particles exceeding 500 nm can be trapped at the injection site. Nanoparticles smaller than 10 nm, or soluble antigen, diffuse into the lymphatic system easily, but their retention time in the lymph nodes is too short to provide sustained antigen delivery. This may explain why OVA pNPs with 500 nm size and soluble OVA are not delivered to the lymph nodes efficiently, while improved NP uptake by lymph node cells is observed for the smaller OVA pNPs. For smaller pNPs sizes, improved uptake is observed for pNP with lower PEG/OVA ratio.

[0168] Therapeutic efficacy of pNPs in a model of melanoma. Encouraged by the fact that OVA pNPs with 10% PEG/OVA ratio result in increased OT-I CD8+ cell proliferation in vitro, improved uptake by APCs (both in vitro and in vivo), and enhanced humoral immune response in vivo, a murine model of B16F10-OVA melanoma was employed to evaluate the therapeutic efficacy of pNPs with a PEG/OVA ratio of 10% compared to solute OVA. Tumor-bearing mice were treated with 10 % PEG/OVA pNPs or solute OVA (10 μ g OVA/ 100 μ L dose), co-administered with CpG (15 μ g/dose). Following the regimen shown in FIG. 9A, C57BL/6 mice (10 mice/treatment group) were inoculated with 1×10^5 B16F10-OVA cells in the SC flank on day 0. Treatments with either 10 % PEG/OVA pNPs or solute OVA were initiated on day 7 after tumor inoculation. A second treatment was given on day 14. Mice were euthanized after their tumors reached 15 mm in any dimension. FIG. 9B shows percentage of animal survival versus days after inoculation of tumor cells. Compared to the no treatment control group, mice treated with solute OVA showed slightly better survival. More than 50 % of mice treated with solute OVA were euthanized due to large tumor burden on day 20, and 100 % of the mice were euthanized on day 21. In contrast, 100 % of mice treated with 10 % PEG/OVA pNPs were alive on day 21 and showed improved survival until the endpoint of the study (day 24).

[0169] In this example, electrohydrodynamic electrospraying is a scalable and versatile nanoparticle manufacturing process, for development of protein-based nanoparticles (pNPs), such as ovalbumin (OVA) pNPs with defined physico-chemical properties. The size or crosslinking agent to protein ratio is a parameter that can determine the immunological responses of pNPs. Specifically, lower PEG/OVA ratios result in softer pNPs with larger mesh sizes, which, in turn, can result in improved CD8+ T cell activation in vitro and improved lymph node drainage and humoral immune response in vivo. Identifying the significance of these parameters allows a pNP formulation to be designed with preclinical potential. In a preclinical murine model of melanoma,

the smaller (200 nm) pNPs of 10% PEG/OVA ratio resulted in improved survival of mice bearing advanced melanoma tumors. It is envisioned that a combination strategy using different types of immunotherapies can be employed. In this case, a combination of OVA pNP administration with adjuvant therapy and immune checkpoint inhibitor therapy could result in further improved preclinical outcomes.

[0170] Example 2

[0171] Size Tunability – Crosslinking Agent Length

[0172] Nanoparticles are collected using endotoxin-free Dulbecco's PBS (EMD Millipore, USA) containing 0.01% tween 20. 5 ml of Endotoxin-free PBS with 0.01% tween 20 is poured into the particle collection plate. A plastic razor blade is used to scratch the surface of the plate and transfer the particles to the buffer. The collected OVA nanoparticles are fully dispersed using a micro-tip sonication, while the tube is submerged in mixture of ice and water in a small beaker to avoid temperature increase caused by tip sonication. The dispersed nanoparticles are passed through a 40 μ m filter. Serial centrifugation steps are used to purify the nanoparticles at the target size. For size separation of 200 nm particles, the filtered solution of nanoparticles are then centrifuged at 4000 rpm for 5 minutes to remove the larger particles. The supernatant that containing the desired size of nanoparticles is centrifuged at 14000 rpm for 60 mins. The pellet is washed with endotoxin free PBS and re-dispersed in 1 ml endotoxin free PBS to be stored at fridge. Samples are made from the stock of nanoparticles for further characterization by dynamic light scattering and BCA assay.

[0173] For 500 nm size separation of OVA nanoparticles, the collected nanoparticles are centrifuged at 1,000 rpm for 1 min. the supernatant is centrifuged for 1 min at 10,000 rpm. The pellet is washed with endotoxin free PBS and re-dispersed in 1 ml endotoxin free PBS to be stored at fridge. Samples are made from the stock of nanoparticles for further characterization by dynamic light scattering and BCA assay.

[0174] Example 3

[0175] Stiffness Tunability – Crosslinking Agent to Protein Ratio

[0176] The chemical amide bond bridging between reactive NHS groups of the crosslinker and amine groups of OVA protein forms a mesh-like structure throughout the stable OVA nanoparticles. To tune the stiffness of nanoparticles, different ratios of crosslinker to protein can be used during the fabrication step. This approach results in producing OVA nanoparticles with different Young's modulus, for example, ranging from 42.7 kPa to 837.8 kPa for 5% crosslinking to 50% crosslinking.

[0177] Atomic force microscopy (AFM). AFM measurements are carried out using an MFP-3D (Oxford Instruments, UK) using CSC-38noAl-A cantilevers (Micromash, USA) with a spring constant of 0.09 N/m. Samples are prepared by electrospraying OVA nanoparticles directly

onto silicon substrates coated with poly(4-Penta fluorophenyl-p-xylylene) via chemical vapor deposition (CVD) polymerization (see supporting information). The substrates are allowed to crosslink at 37 °C for several days prior to use. OVA nanoparticles are localized by scanning the surface in tapping mode over a (5 x 5) μm^2 area and then decreasing the scan area for visualization of a single NP. The force curves are obtained by indenting the tip into the center of an individual nanoparticle and recording the deflection of the cantilever. Through AFM indentation measurements the elastic moduli of OVA nanoparticles are obtained.

[0178] Example 4

[0179] Mesh Size – Crosslinking Density

[0180] As noted above, the mesh size of protein nanoparticles is an important factor affecting controlled release of the cargo from nanocarriers. Employing EHD jetting to fabricate polymerized protein nanoparticles, as is described herein, allows tuning the crosslinking density by simply changing the crosslinker to protein ratio in the particle formulation solution step without the need of any further post-fabrication modification. For OVA nanoparticles, PEG/OVA ratio of 10% and 50% are fabricated. The network density of these two nanoparticle groups are evaluated using small-angle neutron scattering. The results show that average spacing decreases nearly two-fold, forming a more finely divided structure as the PEG/OVA ratio increases from 10% to 50%. Therefore, the protein network becomes more porous and finely heterogeneous with smaller mesh structure with a higher PEG/OVA ratio. The dependency of OVA nanoparticle mesh size on crosslinking agent amount indicates that disclosed electrospraying procedure, in fact, allows for control of the mesh size in the nanoparticles formed.

[0181] Example 5

[0182] Mucin Particles

[0183] By using EHD jetting, with the same procedure disclosed in the present disclosure, mucin-based nanoparticles are fabricated with and without homo-bifunctional PEG-based crosslinker. Mucin protein is dissolved in ultra-pure water at 5 w/v%. In the case of cross-linked mucin particles, a NHS-PEG-NHS crosslinking agent at 10 w/w protein % relative to mucin protein is fully dissolved in water. Then, in both cases, ethylene glycol is added to achieve 80:20 vol. % water-ethylene glycol ratio. Then the final jetting solution is injected through syringes tipped with 26 gauge needles at 0.1 mL/h using a syringe pump. The distance between the tip of the needle and grounded collection plate is 15 cm. The morphology of nanoparticles is characterized using SEM. If NHS-PEG-NHS is used as crosslinker, mucin nanoparticles are kept at 37 °C for 7 days for the completion of crosslinking reaction. Both cross-linked and non-cross-linked mucin nanoparticles are separately collected in PBS. Hydrodynamic diameter and stability

of nanoparticles both with and without crosslinker are measured using DLS. Both approaches resulted in stable mucin nanoparticles.

[0184] Example 6

[0185] Blood Brain Barrier (BBB) Transport – Receptor Targeted Material Choice
5 (Transferrin Particles)

[0186] Instead of modifying nanoparticles with transferrin antibody, by using EHD jetting, nanoparticles are created entirely from transferrin protein that ensures denser presentation of transferrin protein to enhance its recognition by brain endothelial cells. A similar EHD procedure for forming protein nanoparticles discussed in this disclosure is used to fabricate
10 transferrin-based nanoparticles at 200 nm and 500 nm size. In the *in vitro* BBB model using monolayers of human brain endothelium (hCMEC/D3), the uptake and permeability of a library of nanoparticles are tested. The library of nanoparticles includes 50, 100, 200, 500 nm diameter sphere polystyrene nanoparticles, 2AR and 5AR rod-shape polystyrene nanoparticles, 200 and 500 nm transferrin nanoparticles, 200 and 500 nm human serum albumin nanoparticles, 200 and
15 500 nm liposomes. 500 nm transferrin particles show the highest uptake among all 500 nm nanoparticle analyzed and also experience one of the highest particle permeabilities across the BBB model. Interestingly, 200 nm transferrin (TF) nanoparticles show the highest permeabilities among the other 200 nm particles, besides the hydrogel-based particles. This could suggest that 500 nm TF, and 200 nm TF, access a specialized transcytotic pathway, as hCMEC/D3 cells are
20 known to overexpress transferrin receptors.

[0187] Example 7

[0188] Insulin Nanoparticles

[0189] By using EHD jetting, insulin-based nanoparticles are fabricated. Insulin protein at 10 w/v%. and poly(ethylene glycol)-based crosslinker (NHS-PEG-NHS) at 10w/w protein%
25 are prepared in solvent mixture of water, acetic acid, and ethanol with ratios of 80:10:10 vol. %, respectively. Then the final jetting solution flows through syringes tipped with 26-gauge needles at 0.1 mL/h using a syringe pump. The distance between the tip of the needle and grounded collection plate is 15 cm. The morphology of nanoparticles is characterized using SEM. Hydrodynamic diameter and stability of nanoparticles are measured using DLS. This approach
30 results in the formation of stable insulin nanoparticles.

[0190] Example 8

[0191] Lysozyme Nanoparticles

[0192] By using EHD jetting in accordance with certain aspects of the present disclosure, lysozyme-based nanoparticles are fabricated. Lysozyme protein at 10 w/v%. and poly(ethylene glycol)-based crosslinker (NHS-PEG-NHS) at 10w/w protein % are prepared in solvent mixture
35

of water, and ethanol with ratios of 90:10 vol. %, respectively. Then the final jetting solution is flown through syringes tipped with 26-gauge needles at 0.1 mL/h using a syringe pump. The distance between the tip of the needle and grounded collection plate is 15 cm. The morphology of nanoparticles is characterized using SEM. Hydrodynamic diameter and stability of nanoparticles are measured using DLS. This approach resulted in stable lysozyme nanoparticles.

[0193] Example 9

[0194] Hemoglobin Nanoparticles

[0195] By using EHD jetting in accordance with certain aspects of the present disclosure, hemoglobin-based nanoparticles are fabricated. hemoglobin protein at 10 w/v%. and poly(ethylene glycol)-based crosslinker (NHS-PEG-NHS) at 10 w/w protein% are prepared in solvent mixture of water, and ethanol with ratios of 90:10 vol. %, respectively. Then the final jetting solution is injected through syringes tipped with 26-gauge needles at 0.1 mL/h using a syringe pump. The distance between the tip of the needle and grounded collection plate is 15 cm. The morphology of nanoparticles is characterized using SEM. Hydrodynamic diameter and stability of nanoparticles are measured using DLS. This approach resulted in stable hemoglobin nanoparticles.

[0196] Example 10

[0197] Bicompartmental Protein Nanoparticles-Hemoglobin/Insulin Nanoparticles

[0198] By using EHD co-jetting in accordance with certain aspects of the present disclosure, bicompartmentalized hemoglobin/Insulin nanoparticles are fabricated. In electrohydrodynamic co-jetting, two 26G needles are used as capillaries in a side-by-side configuration. The two different protein solutions are pumped at a rate forming laminar flow to ensure a stable interface between the two jetting solutions without any convective mixing. When a droplet is formed at the outlet of the needles, the electric field is applied to the system. Due to rapid evaporation, the initial flow-determined configuration is maintained.

[0199] The two solutions are made as follows. In solution A, hemoglobin protein at 10 w/v%. and poly(ethylene glycol)-based crosslinker (NHS-PEG-NHS) at 10 w/w protein % are prepared in solvent mixture of water, and ethanol with ratios of 90:10 vol. %, respectively. In solution B, insulin protein at 10 w/v % and poly(ethylene glycol)- based crosslinker (NHS-PEG-NHS) at 10w/w protein% is prepared in solvent mixture of water, acetic acid, and ethanol with ratios of 80:10:10 vol. %, respectively. Then one syringe is filled with solution A and the other is filled with solution B. The syringes are then tipped with 26-gauge needles and are placed in a side-by-side configuration in the syringe pump flowing at 0.1 mL/h. The distance between the tip of the needle and grounded collection plate is 15 cm. The applied voltage required to achieve and maintain a stable cone ranged from 10–12 kV. The morphology of nanoparticles is characterized

using SEM and SIM. Hydrodynamic diameter and stability of nanoparticles are measured using DLS. This approach resulted in stable bicompartamental hemoglobin/insulin nanoparticles.

[0200] Example 11

[0201] Albumin-Based Protein Nanoparticles

5 **[0202]** Albumin protein nanoparticles are prepared using EHD jetting. Human serum albumin (HSA) is dissolved in co-solvent system consisting of ultra-pure water (H₂O) and ethylene glycol (EG). Separately, a 2 kDa homo-bifunctional polyethylene glycol crosslinker, O'-bis[2-(N-Succinimidyl-succinylamino)ethyl] polyethylene glycol (NHS-PEG-NHS), is quickly dissolved in ultra-pure water. Combining the two solutions, the final albumin concentration is brought to 7.5 w/v%, while the resulting NHS-PEG-NHS concentration is 10 (w/w protein) % relative to the total albumin. The final co-solvent system comprises an 80:20 vol. % mixture of H₂O:EG. The final protein jetting solution is loaded into a syringe, capped with a 26G stainless steel, blunt-tip needle, and placed into syringe pump to precisely control the flow rate.

15 **[0203]** The positive electrode of a 30 kV power supply is attached to the stainless steel needle, while the aluminum collection surface is grounded and maintained at a distance of 15 cm from the needle tip. The solution is pumped at 0.2 mL/h. Upon the formation of a droplet at the tip of the needle, an electric field is applied, distorting the droplet, forming a Taylor cone. The applied voltage required to achieve and maintain a stable cone ranged from 10–12 kV. Rapid acceleration of a viscoelastic jet in the electric field toward the grounded collection surface leads to size reduction of the resulting jet by several orders of magnitude, facilitating rapid solvent evaporation and solidification of the non-volatile components into spherical nanoparticles. Incorporating the bifunctional NHS ester crosslinker into the jetting solution, the PEG units form stable intermolecular amide bonds through polycondensation reactions with albumin lysine residues. Storage of the nanoparticles in their dry state for a period of seven days at 37°C, completes the crosslinking process, resulting in water-stable, albumin-based protein nanoparticles.

[0204] Example 12

[0205] Tumor-Targeting Protein Nanoparticles

30 **[0206]** Fabrication of tumor-targeting albumin nanoparticles follows a similar approach to that described in Example 7 using EHD jetting in accordance with certain aspects of the present disclosure. In this example, human serum albumin (HSA) and the tumor-targeting, tissue-penetrating peptide, iRGD, are dissolved together in the water and ethylene glycol co-solvent system. Utilizing the same NHS-PEG-NHS crosslinking agent a final solution of protein jetting solution is achieved with a final albumin concentration of 10 w/v%. Similarly, the final NHS-PEG-NHS concentration is 10 w/w% relative to the total albumin concentration. Identical jetting crosslinking conditions are used to generate water-stable, albumin nanoparticles loaded with iRGD.

[0207] Example 13**[0208] siRNA Loaded Protein Nanoparticles**

[0209] Fabrication of short interfering RNA (siRNA) loaded protein nanoparticles followed a similar approach to that of the albumin nanoparticles formed in Example 7 using EHD jetting in accordance with certain aspects of the present disclosure. Here, short interfering sequence of double stranded RNA is first complexed in ultra-pure water with a 60 kDa, branched polyethyleneimine (PEI) for a period of thirty minutes. Following this brief incubation period, the PEI-siRNA solution is added to a previously prepared albumin solution in ultra-pure water and ethylene glycol before being mixed with a separately prepared aqueous NHS- PEG-NHS solution. The final albumin and NHS-PEG crosslinker concentrations are identical to that of the albumin nanoparticle jetting solution previously described. Similar jetting and crosslinking conditions are used to generate siRNA-loaded albumin nanoparticles. In this example, the NHS esters of the homo-bifunctional PEG crosslinker react through polycondensation reactions with both lysine residues of albumin and primary and secondary amines present in the PEI. The result of this process are water-stable, protein nanoparticles designed to release siRNA.

[0210] Example 14**[0211] Paclitaxel-Loaded Albumin Nanoparticles**

[0212] The fabrication of paclitaxel-loaded albumin nanoparticles is performed in a two-step process. First, paclitaxel (PTX) is complexed with soluble albumin protein using high-pressure homogenization. Briefly, PTX is dissolved in DMSO and mixed with an aqueous solution of human serum albumin (HSA). The resulting solution is loaded into a 10 mL syringe. Using a benchtop high-pressure homogenizer, pressures ranging from 12,000-20,000 PSI are applied, forcing the solution through a small orifice creating a homogenized PTX/HSA solution. This process facilitates the loading of PTX into discrete hydrophobic pockets within the protein.

[0213] The homogenization process is repeated until a uniform and opaque solution is obtained. Following this process, the solution is frozen and then lyophilized to yield a dry protein-drug complex. In the second step, following the same process as described previously for the generation of albumin nanoparticles via EHD jetting, a solution is prepared in a water and ethylene glycol cosolvent system. In this example, the protein-drug complex is dissolved in place of the pure albumin with a final albumin-drug concentration of 2.5 wt.%. Identical jetting parameters and crosslinking conditions are used. The result of this combined process are water-stable, PTX-loaded albumin nanoparticles.

[0214] Example 15**[0215] Disulfide Cross-linked Albumin Nanoparticles**

[0216] Albumin protein nanoparticles are prepared using EHD jetting. Human serum albumin (HSA) is dissolved in a (90:10 vol. %) co-solvent system comprising 2,2,2-

trifluoroethanol (TFE) and ultra-pure water (H₂O) at an albumin concentration of 2.5 w/v%. After mixing for a period of thirty minutes, a 10-fold molar excess of β -mercaptoethanol relative to albumin disulfide bonds is added before further mixing the solution for an additional 30 minutes. The incubation of albumin within this solvent system disrupts the normal disulfide bonds present in the protein, creating free cysteine residues, and allowing for a partial denaturing of the protein to take place. The final protein jetting solution is loaded into a syringe, capped with a 26G stainless steel, blunt-tip needle, and placed into syringe pump to precisely control the flow rate. The positive electrode of a 30 kV power supply is attached to the stainless steel needle while the aluminum collection surface is grounded and maintained at a distance of 15 cm from the needle tip. The solution is pumped at 0.2 mL/h. Upon the formation of a droplet at the tip of the needle, an electric field is applied, distorting the droplet, forming a Taylor cone. The applied voltage required to achieve and maintain a stable cone ranged from 5–7 kV. Rapid acceleration of a viscoelastic jet in the electric field toward the grounded collection surface led to size reduction of the resulting jet by several orders of magnitude facilitating rapid solvent evaporation and solidification of the non-volatile components into spherical nanoparticles. Rapid removal of the solvent allows for the reformation of disulfide bonds to occur, both intra- and intermolecularly. The formation of intermolecular disulfide bonds between adjacent albumin molecules within individual particles results in water-stable, disulfide cross-linked protein nanoparticles. In this example, no additional incubation is required to complete the crosslinking process.

[0217] Example 16

[0218] Environment-Reactive Protein Nanoparticles

[0219] To produce particles that are able to sense and react to particular environments, such as a reducing environment, particles are made to be incorporated with a reducible crosslinking, following a similar synthetic scheme as in Example 7 using EHD jetting in accordance with certain aspects of the present disclosure. Human Serum Transferrin is dissolved into a co-solvent system consisting of ultra-pure water (H₂O) and ethanol. 4,7,10,13,16,19,22,25,32,35,38,41,44,47,50,53-Hexadeca-28,29-dithiahexapentacontanedioic acid di-N-succinimidyl ester (Disulfide Crosslinker) Sigma Aldrich, USA) is then separately dissolved into ultra-pure water, and the two solutions combined to bring the solution to a total protein concentration of 10 w/v% and a 10 w/w protein% concentration of Disulfide Crosslinker in relation to total protein content. Identical jetting and crosslinking conditions are then used to result in water-stable nanoparticles. The resulting particles are very similar to those as in Example 7 in regard to morphology and size, but contain a disulfide bond in their crosslinking polymers, which could then be used to release cargos in reducing environments.

[0220] Example 17**[0221] Fast Crosslinking Protein Nanoparticles**

[0222] As a variant of the technology, particles can be made to reduce the crosslinking time. A 10% w/v solution of bovine serum albumin (BSA) is dissolved in co-solvent system comprising of ultra-pure water (H₂O) and ethanol. The solution is then jetted as described in Example. Within a day of jetting (if not done immediately, the particles are stored at 4°C), the particles are placed in a sealed container containing 2.5 mL of 20% glutaraldehyde (v/v in H₂O), and allowed to vapor phase crosslink for 30 minutes. The aldehyde groups in the vapor phase glutaraldehyde are able to react between neighboring amino groups in the proteins, and resulted in stable intermolecular bonds. To quench any unreacted aldehyde groups, which could produce toxicity in *in vivo*, the particles are then immediately collected in PBS containing 100 mM glycine and 0.01% tween20. This results in water stable albumin-based protein nanoparticles.

[0223] Example 18**[0224] Active Therapeutic Enzyme Loaded Protein Nanoparticles**

[0225] To load particles with active therapeutic enzymes, a similar procedure as in Example 13 is used. A 10% w/v solution of a protein blend, which is made of either a 90:10 or 50:50 w/w ratio of HSA and Catalase, is dissolved in co-solvent system comprising ultra-pure water (H₂O) and ethanol. The solution is then jetted, cross-linked and collected as described in Example 13. This results in water stable albumin-based protein nanoparticles loaded with active therapeutic enzymes. The resulting particles are able to process hydrogen peroxide as high levels when compared to unloaded particles, as measured by measuring the reduction of absorbance of hydrogen peroxide at 240 nm using Ultraviolet-visible spectroscopy.

[0226] Example 19**[0227] Biotechnology-Relevant Enzyme Loaded Protein Nanoparticles**

[0228] Enzyme loaded particles protein nanoparticles can be fabricated to contain a biotechnology relevant enzyme such as Horseradish Peroxidase as well. Following a similar synthesis scheme as in Example 14, a 10% w/v solution of a protein blend, in this case a 90:10 w/w ratio of Bovine Serum Albumin and Horseradish Peroxidase, is dissolved in a co-solvent system of ultra-pure water (H₂O) and ethanol. Identical jetting and crosslinking conditions are then used to produce particles, and then collected in a PBS solution containing 100 mM glycine and 0.01% tween20, to quench any unreacted aldehyde groups. This results in water stable albumin-based protein nanoparticles loaded with active biotechnology- relevant enzymes. The activity of the enzymes is shown to be significant as compared to unloaded particles by testing them in a standard TMB assay (Thermo Fisher, USA).

[0229] Example 20**[0230] Protein Nanoparticles with Alkyne-Containing, Trifunctional, PEG-Based Crosslinking Molecule**

[0231] Albumin nanoparticles with an alkyne functional group for post surface
 5 modification are prepared via EHD jetting. The jetting solution is composed of human serum albumin (HSA) at 10 w/v% in a co-solvent system consisting of ultra-pure water and ethylene glycol at 80:20 vol.% ratio. The synthesized trifunctional PEG crosslinker is incorporated into the jetting solution at 10 w/w protein % with respect to the albumin protein. To activate the terminal carboxyl groups of the crosslinker, it is first incubated in water with 5 times molar excess of 1-ethyl-3-(3- dimethylaminopropyl)carbodiimide hydrochloride (EDC) followed by addition of
 10 sulfo-NHS prior being combined with the albumin protein. Then the final jetting solution of protein and crosslinking agent flows through syringes tipped with 26- gauge needles at 0.1 mL/h using a syringe pump. The distance between the tip of the needle and grounded collection plate is 15 cm. To ensure completion of crosslinking reaction, albumin nanoparticles are kept at 37 °C for
 15 7 days. This results in stable, polymerized alkyne modified albumin.

[0232] The foregoing description of the embodiments has been provided for purposes of illustration and description. It is not intended to be exhaustive or to limit the disclosure. Individual elements or features of a particular embodiment are generally not limited to that particular embodiment, but, where applicable, are interchangeable and can be used in a selected
 20 embodiment, even if not specifically shown or described. The same may also be varied in many ways. Such variations are not to be regarded as a departure from the disclosure, and all such modifications are intended to be included within the scope of the disclosure.

VARIOUS EMBODIMENTS

Some embodiments of the technology described herein can be defined according to any of
 25 the following numbered embodiments:

1. A nanoparticle comprising a cross-linked water-soluble protein having an average molecular weight of greater than or equal to about 8 kDa and less than or equal to about 700 kDa and defining a mesh structure having an average linear mesh size of greater than or equal to about 1 nm to less than or equal to about 4 nm.
- 30 2. The nanoparticle of embodiment 1 further comprising a crosslinking agent reacted with the water-soluble protein.
3. The nanoparticle of embodiment 2, wherein the cross-linked water soluble protein is present at greater than or equal to about 50% by weight to less than or equal to about 95% by

weight and the crosslinking agent is present at greater than or equal to about 5% by weight to less than or equal to about 50% by weight.

4. The nanoparticle of embodiment 2, wherein prior to reacting with the water-soluble protein, the crosslinking agent comprises a reactive group selected from the group consisting of:
5 an alkenyl group, an alkynyl group, a maleimide group, an active ester group, an anhydride group, an N-succinimidyl group, a triflate group, and combinations thereof.

5. The nanoparticle of embodiment 1 further comprising one or more of a therapeutic active ingredient, an imaging agent, and a targeting moiety.

6. The nanoparticle of embodiment 1, wherein the water-soluble protein is selected
10 from the group consisting of: ovalbumin, albumin, mucin, transferrin, insulin, lysozyme, hemoglobin, collagen, and combinations thereof.

7. A method of making a nanoparticle comprising:
jetting a liquid comprising a water-soluble protein having an average molecular weight of
greater than or equal to about 8 kDa and less than or equal to about 700 kDa and water through a
15 nozzle; and

exposing the liquid to an electric field sufficient to solidify the liquid and form the nanoparticle defining a mesh structure having an average linear mesh size of greater than or equal to about 1 nm to less than or equal to about 4 nm.

8. The method of embodiment 7, wherein the liquid further comprises a crosslinking
20 agent and during the exposing, the water-soluble protein is at least partially cross-linked.

9. The method of embodiment 8, wherein the at least partially cross-linked water-soluble protein defines a mesh structure having an average linear mesh size of greater than or equal to about 1 nm to less than or equal to about 4 nm.

10. The method of embodiment 7, wherein the electric field is formed by applying a
25 potential difference between at least two electrodes from about 0.1 kV to about 25 kV.

11. The method of embodiment 7, wherein the liquid further comprises an additive selected from the group consisting of: a therapeutic active ingredient, an imaging agent, a biomolecule, a targeting moiety, and combinations thereof, wherein the additive is incorporated into the nanoparticle.

30 12. A nanoparticle comprising a cross-linked water-soluble protein having an average molecular weight of greater than or equal to about 8 kDa to less than or equal to about 700 kDa and comprising disulfide bonds, wherein the nanoparticle is substantially free of a distinct crosslinking agent.

13. The nanoparticle of embodiment 12, wherein the cross-linked water-soluble
35 protein defines a mesh structure.

14. The nanoparticle of embodiment 12, wherein the cross-linked water-soluble protein is selected from the group consisting of: ovalbumin, albumin, human serum albumin, bovine serum albumin, transferrin, hemoglobin, IgG, enzymes, transport proteins, storage proteins, antibodies, aptamers, chemokines, hormonal proteins, polypeptides, and combinations thereof.

5 15. The nanoparticle of embodiment 12 further comprising one or more of a therapeutic active ingredient, an imaging agent, a biomolecule, and a targeting moiety.

16. A method of making a nanoparticle comprising:

jetting a liquid comprising a water-soluble protein having an average molecular weight of greater than or equal to about 8 kDa to less than or equal to about 700 kDa and comprising
10 disulfide bonds through a nozzle; and

exposing the liquid to an electric field sufficient to cross-link and solidify the liquid and form the nanoparticle that is substantially free of a distinct crosslinking agent.

17. The method of embodiment 16, wherein the water-soluble protein defines a mesh structure having an average linear mesh size of greater than or equal to about 1 nm to less than or
15 equal to about 4 nm.

18. The method of embodiment 16, wherein the electric field is formed by applying a potential difference between at least two electrodes from about 0.1 kV to about 25 kV.

19. The method of embodiment 16, wherein the liquid further comprises an additive selected from the group consisting of: a therapeutic active ingredient, an imaging agent, a
20 biomolecule, a targeting moiety, and combinations thereof, wherein the additive is incorporated into the nanoparticle.

20. The method of embodiment 16, wherein the water-soluble protein is selected from the group consisting of: ovalbumin, albumin, human serum albumin, bovine serum albumin, transferrin, hemoglobin, IgG, enzymes, transport proteins, storage proteins, antibodies, aptamers,
25 chemokines, hormonal proteins, polypeptides, and combinations thereof.

21. A multicompartmental nanoparticle comprising:

a first compartment defining at least a portion of an exposed surface of the multicompartmental nanoparticle and comprising a first composition having a water-soluble polymer having an average molecular weight of greater than or equal to about 8 kDa and less than
30 or equal to about 700 kDa; and

at least one additional compartment defining at least a portion of an exposed surface and comprising at least one additional composition distinct from the first composition.

22. The multicompartmental nanoparticle of embodiment 21, further comprising a crosslinking agent reacted with the water-soluble protein in the first compartment.

23. The multicompartmental nanoparticle of embodiment 22, wherein the water-soluble protein is present at greater than or equal to about 50% by weight to less than or equal to about 95% by weight and the crosslinking agent is present at greater than or equal to about 5% by weight to less than or equal to about 50% by weight.

5 24. The multicompartmental nanoparticle of embodiment 22, wherein prior to reacting with the water-soluble protein, the crosslinking agent comprises a reactive group selected from the group consisting of: an alkenyl group, an alkynyl group, a maleimide group, an active ester group, an anhydride group, an N-succinimidyl group, a triflate group, and combinations thereof.

10 25. The multicompartmental nanoparticle of embodiment 21, further comprising one or more of a therapeutic active ingredient, an imaging agent, a biomolecule, and a targeting moiety.

26. The multicompartmental nanoparticle of embodiment 21, wherein the water-soluble protein is selected from the group consisting of: ovalbumin, albumin, mucin, transferrin, insulin, lysozyme, hemoglobin, collagen, and combinations thereof.

15 27. The multicompartmental nanoparticle of embodiment 21 where the water-soluble protein is a first water soluble protein and the at least one additional compartment comprises a second water-soluble protein.

28. The multicompartmental nanoparticle of embodiment 27, wherein the second water-soluble protein is selected from the group consisting of: ovalbumin, albumin, mucin, transferrin, insulin, lysozyme, hemoglobin, collagen, and combinations thereof.

20 29. A nanoparticle comprising a cross-linked water-soluble protein having an average molecular weight of greater than or equal to about 8 KDa and less than or equal to about 700 kDa and comprising a therapeutic active ingredient.

25 30. The nanoparticle of embodiment 29, wherein the therapeutic active ingredient is selected from the group consisting of: DNA, RNA, plasmids, short interfering sequence of double stranded RNA (siRNA), messenger RNA (mRNA), transfer RNA, ribosomal RNA, small nuclear RNA, single stranded DNA, CRISPR CAS-9, aptamers, antibodies, peptides, targeting molecules, vitamins, and combinations thereof.

31. The nanoparticle of embodiment 29 further comprising a crosslinking agent reacted with the water-soluble protein.

30 32. The nanoparticle of embodiment 29, wherein the cross-linked water soluble protein is present at greater than or equal to about 50% by weight to less than or equal to about 95% by weight and the crosslinking agent is present at greater than or equal to about 5% by weight to less than or equal to about 50% by weight.

35 33. The nanoparticle of embodiment 29, wherein prior to reacting with the water-soluble protein, the crosslinking agent comprises a reactive group selected from the group

consisting of: an alkenyl group, an alkynyl group, a maleimide group, an active ester group, an anhydride group, an N-succinimidyl group, a triflate group, and combinations thereof.

34. The nanoparticle of embodiment 29, further comprising one or more of an imaging agent, an additional biomolecule, and a targeting moiety.

5 35. The nanoparticle of embodiment 29, wherein the therapeutic active ingredient is selected from the group consisting of: a drug, a steroid, and combinations thereof.

36. The nanoparticle of embodiment 35, wherein the drug is selected from the group consisting of: paclitaxel, cis-platin, doxorubicin, and combinations thereof.

10 37. The nanoparticle of embodiment 29, wherein the therapeutic active ingredient is selected from the group consisting of: an antibody, an aptamer, a chemokine, a peptide drug, and combinations thereof.

38. The nanoparticle of embodiment 29, wherein the water-soluble protein is selected from the group consisting of: ovalbumin, albumin, mucin, transferrin, insulin, lysozyme, hemoglobin, collagen, and combinations thereof.

15 39. In certain aspects, the present disclosure relates to a nanoparticle comprising a cross-linked water-soluble protein having an average molecular weight of greater than or equal to about 8 kDa and less than or equal to about 700 kDa (e.g., from 8 kDa to 15 kDa, from 10 kDa to 20 kDa, from 15 kDa to 25 kDa, from 25 kDa to 50 kDa, from 50 kDa to 100 kDa, from 100 kDa to 200 kDa, from 200 kDa to 300 kDa, from 300 kDa to 400 kDa, from 400 kDa to 500 kDa, from 20 500 kDa to 600 kDa, or from 600 kDa to 700 kDa). In some embodiments, the nanoparticle has a mesh structure with an average linear mesh size of greater than or equal to about 1 nm to less than or equal to about 4 nm (e.g., from 1 nm to 3 nm, or from 2 nm to 4 nm, e.g., from 1 nm to 2 nm, from 2 nm to 3 nm, or from 3 nm to 4 nm, e.g., about 1 nm, about 2 nm, about 3 nm, or about 4 nm).

25 40. In some aspects, the nanoparticle further comprises a crosslinking agent reacted with (e.g., conjugated to) the water-soluble protein.

41. In some aspects, the cross-linked water soluble protein is present at greater than or equal to about 50% by weight to less than or equal to about 95% by weight (e.g., about 50% by weight, about 55% by weight, about 60% by weight, about 65% by weight, about 70% by weight, about 75% by weight, about 80% by weight, about 85% by weight, about 90% by weight, or about 30 95% by weight) (e.g., dry weight) of the nanoparticle and the crosslinking agent is present at greater than or equal to about 5% by weight to less than or equal to about 50% by weight (e.g., about 5% by weight, about 10% by weight, about 15% by weight, about 20% by weight, about 25% by weight, about 30% by weight, about 35% by weight, about 40% by weight, about 45% by weight, or about 50% by weight) (e.g., dry weight) of the nanoparticle.

42. In some aspects, prior to reacting with the water-soluble protein, the crosslinking agent comprises a reactive group selected from the group consisting of an alkenyl group, an alkynyl group, a maleimide group, an active ester group, an anhydride group, an N-succinimidyl group, a triflate group, and combinations thereof.

5 43. In some aspects, the nanoparticle further comprises one or more of a therapeutic active ingredient (e.g., a biomolecule, e.g., a nucleic acid, e.g., DNA), an imaging agent, and a targeting moiety.

44. In some aspects, the water-soluble protein is selected from the group consisting of albumin, ovalbumin, mucin, transferrin, insulin, lysozyme, hemoglobin, collagen, and
10 combinations thereof.

45. In another aspect, the invention provides a method of treating a subject having a cancer (e.g., glioblastoma), the method including administering to the subject the nanoparticle of any one of the preceding embodiments in an effective amount to treat the cancer (e.g., glioblastoma).

46. In another aspect, the invention provides a pharmaceutical composition including
15 the nanoparticle of any one of the preceding embodiments.

47. In certain aspects, the present disclosure also relates to a method of making a nanoparticle that comprises jetting a liquid comprising a water-soluble protein having an average molecular weight of greater than or equal to about 8 kDa and less than or equal to about 700 kDa (e.g., from 8 kDa to 15 kDa, from 10 kDa to 20 kDa, from 15 kDa to 25 kDa, from 25 kDa to 50
20 kDa, from 50 kDa to 100 kDa, from 100 kDa to 200 kDa, from 200 kDa to 300 kDa, from 300 kDa to 400 kDa, from 400 kDa to 500 kDa, from 500 kDa to 600 kDa, or from 600 kDa to 700 kDa) and water (e.g., water containing one or more solutes, e.g., buffers) through a nozzle. The method also comprises exposing the liquid to an electric field sufficient to solidify the liquid and form the nanoparticle. The nanoparticle has a mesh structure having an average linear mesh size
25 of greater than or equal to about 1 nm to less than or equal to about 4 nm (e.g., from 1 nm to 3 nm, or from 2 nm to 4 nm, e.g., from 1 nm to 2 nm, from 2 nm to 3 nm, or from 3 nm to 4 nm, e.g., about 1 nm, about 2 nm, about 3 nm, or about 4 nm).

48. In some aspects, the liquid further comprises a crosslinking agent and during the exposing, the water-soluble protein is at least partially cross-linked.

30 49. In some aspects, the at least partially cross-linked water-soluble protein defines a mesh structure having an average linear mesh size of greater than or equal to about 1 nm to less than or equal to about 4 nm (e.g., from 1 nm to 3 nm, or from 2 nm to 4 nm, e.g., from 1 nm to 2 nm, from 2 nm to 3 nm, or from 3 nm to 4 nm, e.g., about 1 nm, about 2 nm, about 3 nm, or about 4 nm).

50. In some aspects, the electric field is formed by applying a potential difference
35 between at least two electrodes from about 0.1 kV to about 25 kV (e.g., from about 0.1 kV to

about 0.5 kV, from about 0.5 kV to about 1.0 kV, from about 1.0 kV to about 5 kV, from about 5 kV to about 10 kV, from about 10 kV to about 15 kV, from about 15 kV to about 20 kV, or from about 20 kV to about 25 kV, e.g., about 0.1 kV, about 0.5 kV, about 1.0 kV, about 2.0 kV, about 5.0 kV, about 10 kV, about 15 kV, about 20 kV, or about 25 kV).

5 51. In some aspects, the liquid further comprises an additive selected from the group consisting of a therapeutic active ingredient, an imaging agent, a biomolecule, a targeting moiety, and a combination thereof, wherein the additive is incorporated into the nanoparticle.

 52. In certain other aspects, the present disclosure relates to a nanoparticle comprising a cross-linked water-soluble protein having an average molecular weight of greater than or equal
10 to about 8 kDa to less than or equal to about 700 kDa (e.g., from 8 kDa to 15 kDa, from 10 kDa to 20 kDa, from 15 kDa to 25 kDa, from 25 kDa to 50 kDa, from 50 kDa to 100 kDa, from 100 kDa to 200 kDa, from 200 kDa to 300 kDa, from 300 kDa to 400 kDa, from 400 kDa to 500 kDa, from 500 kDa to 600 kDa, or from 600 kDa to 700 kDa). The cross-linked water-soluble protein comprises disulfide bonds. The nanoparticle is also substantially free of a distinct crosslinking agent.

15 53. In some aspects, the cross-linked water-soluble protein defines a mesh structure.

 54. In some aspects, the cross-linked water-soluble protein is selected from the group consisting of albumin, human serum albumin, ovalbumin, bovine serum albumin, transferrin, hemoglobin, IgG, enzymes, transport proteins, storage proteins, antibodies, aptamers, chemokines, hormonal proteins, polypeptides, and combinations thereof.

20 55. In some aspects, the nanoparticle further comprises one or more of a therapeutic active ingredient (e.g., a biomolecule, e.g., a nucleic acid, e.g., DNA), an imaging agent, and a targeting moiety.

 56. In yet other aspects, the present disclosure relates to a method of making a nanoparticle comprising: jetting a liquid comprising a water-soluble protein having an average
25 molecular weight of greater than or equal to about 8 kDa to less than or equal to about 700 kDa (e.g., from 8 kDa to 15 kDa, from 10 kDa to 20 kDa, from 15 kDa to 25 kDa, from 25 kDa to 50 kDa, from 50 kDa to 100 kDa, from 100 kDa to 200 kDa, from 200 kDa to 300 kDa, from 300 kDa to 400 kDa, from 400 kDa to 500 kDa, from 500 kDa to 600 kDa, or from 600 kDa to 700 kDa) through a nozzle. The water-soluble protein comprises disulfide bonds. The method also
30 comprises exposing the liquid to an electric field sufficient to cross-link and solidify the liquid and form the nanoparticle substantially free of a distinct crosslinking agent.

 57. In some aspects, the water-soluble protein has a mesh structure having an average linear mesh size of greater than or equal to about 1 nm to less than or equal to about 4 nm (e.g., from 1 nm to 3 nm, or from 2 nm to 4 nm, e.g., from 1 nm to 2 nm, from 2 nm to 3 nm, or from 3
35 nm to 4 nm, e.g., about 1 nm, about 2 nm, about 3 nm, or about 4 nm).

58. In some aspects, the electric field is formed by applying a potential difference between at least two electrodes from about 0.1 kV to about 25 kV (e.g., from about 0.1 kV to about 0.5 kV, from about 0.5 kV to about 1.0 kV, from about 1.0 kV to about 5 kV, from about 5 kV to about 10 kV, from about 10 kV to about 15 kV, from about 15 kV to about 20 kV, or from about 20 kV to about 25 kV, e.g., about 0.1 kV, about 0.5 kV, about 1.0 kV, about 2.0 kV, about 5.0 kV, about 10 kV, about 15 kV, about 20 kV, or about 25 kV).

59. In some aspects, the liquid further comprises an additive selected from the group consisting of a therapeutic active ingredient, an imaging agent, a biomolecule, a targeting moiety, and combinations thereof, wherein the additive is incorporated into the nanoparticle.

60. In some aspects, the water-soluble protein is selected from the group consisting of ovalbumin, albumin, human serum albumin, bovine serum albumin, transferrin, hemoglobin, IgG, enzymes, transport proteins, storage proteins, antibodies, aptamers, chemokines, hormonal proteins, polypeptides, and combinations thereof.

61. In yet other aspects, the present disclosure relates to a multicompartmental nanoparticle comprising a first compartment defining at least a portion of an exposed surface of the multicompartmental nanoparticle and comprising a first composition having a water-soluble polymer with an average molecular weight of greater than or equal to about 8 kDa and less than or equal to about 700 kDa (e.g., from 8 kDa to 15 kDa, from 10 kDa to 20 kDa, from 15 kDa to 25 kDa, from 25 kDa to 50 kDa, from 50 kDa to 100 kDa, from 100 kDa to 200 kDa, from 200 kDa to 300 kDa, from 300 kDa to 400 kDa, from 400 kDa to 500 kDa, from 500 kDa to 600 kDa, or from 600 kDa to 700 kDa). The multicompartmental nanoparticle also comprises at least one additional compartment constituting (e.g., defining) at least a portion of an exposed surface and comprising at least one additional composition distinct from the first composition.

62. In some aspects, the multicompartmental nanoparticle further comprises a crosslinking agent reacted with the water-soluble protein in the first compartment.

63. In some aspects, the water soluble protein is present at greater than or equal to about 50% by weight to less than or equal to about 95% by weight (e.g., about 50% by weight, about 55% by weight, about 60% by weight, about 65% by weight, about 70% by weight, about 75% by weight, about 80% by weight, about 85% by weight, about 90% by weight, or about 95% by weight) (e.g., dry weight) of the nanoparticle and the crosslinking agent is present at greater than or equal to about 5% by weight to less than or equal to about 50% by weight (e.g., about 5% by weight, about 10% by weight, about 15% by weight, about 20% by weight, about 25% by weight, about 30% by weight, about 35% by weight, about 40% by weight, about 45% by weight, or about 50% by weight) (e.g., dry weight) of the nanoparticle.

64. In some aspects, prior to reacting with the water-soluble protein, the crosslinking agent comprises a reactive group selected from the group consisting of: an alkenyl group, an alkynyl group, a maleimide group, an active ester group, an anhydride group, an N-succinimidyl group, a triflate group, and combinations thereof.

5 65. In some aspects, the multicompartmental nanoparticle further comprises one or more of a therapeutic active ingredient, an imaging agent, a biomolecule, and a targeting moiety.

66. In some aspects, the water-soluble protein is selected from the group consisting of albumin, ovalbumin, mucin, transferrin, insulin, lysozyme, hemoglobin, collagen, and combinations thereof.

10 67. In some aspects, the water-soluble protein is a first water soluble protein and the at least one additional compartment comprises a second water-soluble protein.

68. In some aspects, the second water-soluble protein is selected from the group consisting of albumin, ovalbumin, mucin, transferrin, insulin, lysozyme, hemoglobin, collagen, and combinations thereof.

15 69. In another aspect, the invention provides a method of treating a subject having a cancer (e.g., glioblastoma), the method including administering to the subject the multicompartmental nanoparticle of any one of the preceding embodiments in an effective amount to treat the cancer (e.g., glioblastoma).

20 70. In another aspect, the invention provides a pharmaceutical composition including the multicompartmental nanoparticle of any one of the preceding embodiments.

71. In certain aspects, the present disclosure also relates to a nanoparticle comprising a cross-linked water-soluble protein having an average molecular weight of greater than or equal to about 8 kDa and less than or equal to about 700 kDa (e.g., from 8 kDa to 15 kDa, from 10 kDa to 20 kDa, from 15 kDa to 25 kDa, from 25 kDa to 50 kDa, from 50 kDa to 100 kDa, from 100 kDa to 200 kDa, from 200 kDa to 300 kDa, from 300 kDa to 400 kDa, from 400 kDa to 500 kDa, from 500 kDa to 600 kDa, or from 600 kDa to 700 kDa). In some embodiments, the nanoparticle also comprises a therapeutic active ingredient (e.g., a biomolecule, e.g., a nucleic acid, e.g., DNA).

72. In some aspects, the therapeutic active ingredient is selected from the group consisting of a nucleic acid (e.g., DNA, RNA, plasmid, short interfering sequence of double stranded RNA (siRNA, e.g., siRNA against STAT3), messenger RNA (mRNA), transfer RNA, ribosomal RNA, small nuclear RNA, single stranded DNA, CRISPR CAS-9, or aptamer), a protein or peptide (e.g., an antibody or other targeting molecule), a vitamin, and a combination thereof.

73. In some aspects, the nanoparticle further comprises a crosslinking agent reacted with the water-soluble protein.

74. In some aspects, the water soluble protein is present at (e.g., accounts for) greater than or equal to about 50% by weight to less than or equal to about 95% by weight (e.g., about 50% by weight, about 55% by weight, about 60% by weight, about 65% by weight, about 70% by weight, about 75% by weight, about 80% by weight, about 85% by weight, about 90% by weight, or about 95% by weight) (e.g., dry weight) of the nanoparticle and the crosslinking agent is present at (e.g., accounts for) greater than or equal to about 5% by weight to less than or equal to about 50% by weight (e.g., about 5% by weight, about 10% by weight, about 15% by weight, about 20% by weight, about 25% by weight, about 30% by weight, about 35% by weight, about 40% by weight, about 45% by weight, or about 50% by weight) (e.g., dry weight) of the nanoparticle.

75. In some aspects, prior to reacting with the water-soluble protein, the crosslinking agent comprises a reactive group selected from the group consisting of an alkenyl group, an alkynyl group, a maleimide group, an active ester group, an anhydride group, an N-succinimidyl group, a triflate group, and a combinations thereof.

76. In some aspects, the nanoparticle further comprises one or more of an imaging agent, an additional biomolecule, and a targeting moiety.

77. In some aspects, the therapeutic active ingredient is selected from the group consisting of a drug, a steroid, and combinations thereof.

78. In some aspects, the drug is selected from the group consisting of: paclitaxel, cis-platin, doxorubicin, and combinations thereof.

79. In some aspects, the therapeutic active ingredient is selected from the group consisting of an antibody, an aptamer, a chemokine, a peptide drug, and combinations thereof.

80. In some aspects, the water-soluble protein is selected from the group consisting of albumin, ovalbumin, mucin, transferrin, insulin, lysozyme, hemoglobin, collagen, and combinations thereof.

81. In another aspect, the invention provides a method of treating a subject having a cancer (e.g., glioblastoma), the method including administering to the subject the nanoparticle of any one of the preceding embodiments in an effective amount to treat the cancer (e.g., glioblastoma).

82. In another aspect, the invention provides a pharmaceutical composition including the nanoparticle of any one of the preceding embodiments.

CLAIMS

What is claimed is:

1. A nanoparticle comprising a cross-linked water-soluble protein having a mesh structure, wherein the water-soluble protein has an average molecular weight of greater than or equal to about 8 kDa and less than or equal to about 700 kDa.
2. The nanoparticle of claim 1, wherein the mesh structure has an average linear mesh size of greater than or equal to about 1 nm to less than or equal to about 4 nm.
3. The nanoparticle of claim 1 or 2, wherein the nanoparticle comprises a crosslinking agent conjugated to the water-soluble protein.
4. The nanoparticle of claim 3, wherein the cross-linked water soluble protein is present at greater than or equal to about 50% by weight to less than or equal to about 95% by weight, and wherein the crosslinking agent is present at greater than or equal to about 5% by weight to less than or equal to about 50% by weight.
5. The nanoparticle of claim 3 or 4, wherein prior to reacting with the water-soluble protein, the crosslinking agent comprises a reactive group selected from the group consisting of an alkenyl group, an alkynyl group, a maleimide group, an active ester group, an anhydride group, an N-succinimidyl group, a triflate group, and a combination thereof.
6. The nanoparticle of any one of claims 3-5, wherein the crosslinking agent is a homo-bifunctional polymer.
7. The nanoparticle of any one of claims 1-6, further comprising one or more of a therapeutic active ingredient, an imaging agent, and a targeting moiety.
8. The nanoparticle of claim 7, wherein the nanoparticle comprises a therapeutic active ingredient which is a biomolecule.
9. The nanoparticle of claim 8, wherein the biomolecule is a nucleic acid.
10. The nanoparticle of claim 8, wherein the biomolecule is DNA.
11. The nanoparticle of any one of claims 1-10, wherein the water-soluble protein is selected from the group consisting of albumin, ovalbumin, mucin, transferrin, insulin, lysozyme, hemoglobin, collagen, and a combination thereof.

12. A method of treating a subject having a cancer, the method comprising administering to the subject the nanoparticle of any one of claims 1-11 in an effective amount to treat the cancer.

13. A pharmaceutical composition comprising the nanoparticle of any one of claims 1-11.

5 14. A method of making a nanoparticle comprising:
jetting a liquid comprising a water-soluble protein having an average molecular weight of greater than or equal to about 8 kDa and less than or equal to about 700 kDa and water through a nozzle; and

10 exposing the liquid to an electric field sufficient to solidify the liquid and form the nanoparticle defining a mesh structure having an average linear mesh size of greater than or equal to about 1 nm to less than or equal to about 4 nm.

15 15. The method of claim 14, wherein the liquid further comprises a crosslinking agent and during the exposing, the water-soluble protein is at least partially cross-linked.

16. The method of claim 15, wherein the at least partially cross-linked water-soluble protein defines a mesh structure having an average linear mesh size of greater than or equal to about 1 nm to less than or equal to about 4 nm.

17. The method of any one of claims 14-16, wherein the electric field is formed by applying a potential difference between at least two electrodes from about 0.1 kV to about 25 kV.

20 18. The method of any one of claims 14-17, wherein the liquid further comprises an additive selected from the group consisting of a therapeutic active ingredient, an imaging agent, a targeting moiety, and a combination thereof, wherein the additive is incorporated into the nanoparticle.

19. The method of claim 18, wherein the additive is a therapeutic active ingredient which is a biomolecule.

20. The method of claim 19, wherein the biomolecule is a nucleic acid.

25 21. The method of claim 19, wherein the biomolecule is DNA.

22. A nanoparticle comprising a cross-linked water-soluble protein having an average molecular weight of greater than or equal to about 8 kDa to less than or equal to about 700 kDa and comprising disulfide bonds, wherein the nanoparticle is substantially free of a distinct crosslinking agent.

23. The nanoparticle of claim 22, wherein the cross-linked water-soluble protein defines a mesh structure.

24. The nanoparticle of claim 23, wherein the cross-linked water-soluble protein is selected from the group consisting of albumin, human serum albumin, ovalbumin, bovine serum
5 albumin, transferrin, hemoglobin, IgG, enzymes, transport proteins, storage proteins, antibodies, aptamers, chemokines, hormonal proteins, polypeptides, and combinations thereof.

25. The nanoparticle of any one of claims 22-24, further comprising one or more of a therapeutic active ingredient, an imaging agent, a biomolecule, and a targeting moiety.

26. A method of treating a subject having a cancer, the method comprising
10 administering to the subject the nanoparticle of any one of claims 22-25 in an effective amount to treat the cancer.

27. A pharmaceutical composition comprising the nanoparticle of any one of claims 22-25.

28. A method of making a nanoparticle comprising:
15 jetting a liquid comprising a water-soluble protein having an average molecular weight of greater than or equal to about 8 kDa to less than or equal to about 700 kDa and comprising disulfide bonds through a nozzle; and

exposing the liquid to an electric field sufficient to cross-link and solidify the liquid and form the nanoparticle that is substantially free of a distinct crosslinking agent.

20 29. The method of claim 26, wherein the water-soluble protein defines a mesh structure having an average linear mesh size of greater than or equal to about 1 nm to less than or equal to about 4 nm.

30. The method of claim 28 or 29, wherein the electric field is formed by applying a potential difference between at least two electrodes from about 0.1 kV to about 25 kV.

25 31. The method of any one of claims 28-30, wherein the liquid further comprises an additive selected from the group consisting of a therapeutic active ingredient, an imaging agent, a targeting moiety, and a combination thereof, wherein the additive is incorporated into the nanoparticle.

32. The method of claim 20, wherein the therapeutic active ingredient is a biomolecule.

33. The method of claim 32, wherein the biomolecule is a nucleic acid.

34. The method of claim 32, wherein the biomolecule is DNA.

35. The method of any one of claims 28-34, wherein the water-soluble protein is selected from the group consisting of albumin, human serum albumin, ovalbumin, bovine serum albumin, transferrin, hemoglobin, IgG, an enzyme, a transport protein, a storage protein, an antibody, an aptamer, a chemokines, a hormonal protein, a polypeptide, and a combination thereof.

36. A multicompartmental nanoparticle comprising:

a first compartment defining at least a portion of an exposed surface of the multicompartmental nanoparticle and comprising a first composition having a water-soluble polymer having an average molecular weight of greater than or equal to about 8 kDa and less than or equal to about 700 kDa; and

at least one additional compartment defining at least a portion of an exposed surface and comprising at least one additional composition distinct from the first composition.

37. The multicompartmental nanoparticle of claim 36, further comprising a crosslinking agent conjugated to the water-soluble protein in the first compartment.

38. The multicompartmental nanoparticle of claim 37, wherein the water-soluble protein is present at greater than or equal to about 50% by weight to less than or equal to about 95% by weight and the crosslinking agent is present at greater than or equal to about 5% by weight to less than or equal to about 50% by weight.

39. The multicompartmental nanoparticle of claim 37 or 38, wherein prior to reacting with the water-soluble protein, the crosslinking agent comprises a reactive group selected from the group consisting of an alkenyl group, an alkynyl group, a maleimide group, an active ester group, an anhydride group, an N-succinimidyl group, a triflate group, and combinations thereof.

40. The multicompartmental nanoparticle of any one of claims 36-39, further comprising one or more of a therapeutic active ingredient, an imaging agent, and a targeting moiety.

41. The multicompartmental nanoparticle of claim 40, wherein the multicompartmental nanoparticle comprises a therapeutic active ingredient which is a biomolecule.

42. The multicompartmental nanoparticle of claim 41, wherein the biomolecule is a nucleic acid.

43. The multicompartmental nanoparticle of claim 41, wherein the biomolecule is DNA.

44. The multicompartmental nanoparticle of any one of claims 36-43, wherein the water-soluble protein is selected from the group consisting of albumin, ovalbumin, mucin, transferrin, insulin, lysozyme, hemoglobin, collagen, and a combination thereof.

5 45. The multicompartmental nanoparticle of any one of claims 36-44, wherein the water-soluble protein is a first water soluble protein and the at least one additional compartment comprises a second water-soluble protein.

46. The multicompartmental nanoparticle of claim 45, wherein the second water-soluble protein is selected from the group consisting of albumin, ovalbumin, mucin, transferrin,
10 insulin, lysozyme, hemoglobin, collagen, and a combination thereof.

47. A method of treating a subject having a cancer, the method comprising administering to the subject the multicompartmental nanoparticle of any one of claims 36-46 in an effective amount to treat the cancer.

48. A pharmaceutical composition comprising the multicompartmental nanoparticle of
15 any one of claims 36-46.

49. A nanoparticle comprising a cross-linked water-soluble protein having an average molecular weight of greater than or equal to about 8 kDa and less than or equal to about 700 kDa and a therapeutic active ingredient.

50. The nanoparticle of claim 49, wherein the therapeutic active ingredient is selected
20 from the group consisting of DNA, RNA, plasmids, short interfering sequence of double stranded RNA (siRNA), messenger RNA (mRNA), transfer RNA, ribosomal RNA, small nuclear RNA, single stranded DNA, CRISPR CAS-9, aptamers, antibodies, peptides, targeting molecules, vitamins, and combinations thereof.

51. The nanoparticle of claim 49 or 50, further comprising a crosslinking agent
25 conjugated to the water-soluble protein.

52. The nanoparticle of any one of claims 49-51, wherein the cross-linked water soluble protein is present at greater than or equal to about 50% by weight to less than or equal to about 95% by weight and the crosslinking agent is present at greater than or equal to about 5% by weight to less than or equal to about 50% by weight.

53. The nanoparticle of any one of claims 49-52, wherein prior to reacting with the water-soluble protein, the crosslinking agent comprises a reactive group selected from the group consisting of an alkenyl group, an alkynyl group, a maleimide group, an active ester group, an anhydride group, an N-succinimidyl group, a triflate group, and a combination thereof.

5 54. The nanoparticle of any one of claims 49-53, further comprising one or more of an imaging agent, an additional biomolecule, and a targeting moiety.

55. The nanoparticle of any one of claims 49-54, wherein the therapeutic active ingredient is selected from the group consisting of a drug, a steroid, and combinations thereof.

10 56. The nanoparticle of claim 55, wherein the drug is selected from the group consisting of: paclitaxel, cis-platin, doxorubicin, and combinations thereof.

57. The nanoparticle of claim 49, wherein the therapeutic active ingredient is selected from the group consisting of: an antibody, an aptamer, a chemokine, a peptide drug, and combinations thereof.

15 58. The nanoparticle of any one of claims 49-57, wherein the water-soluble protein is selected from the group consisting of albumin, ovalbumin, mucin, transferrin, insulin, lysozyme, hemoglobin, collagen, and a combination thereof.

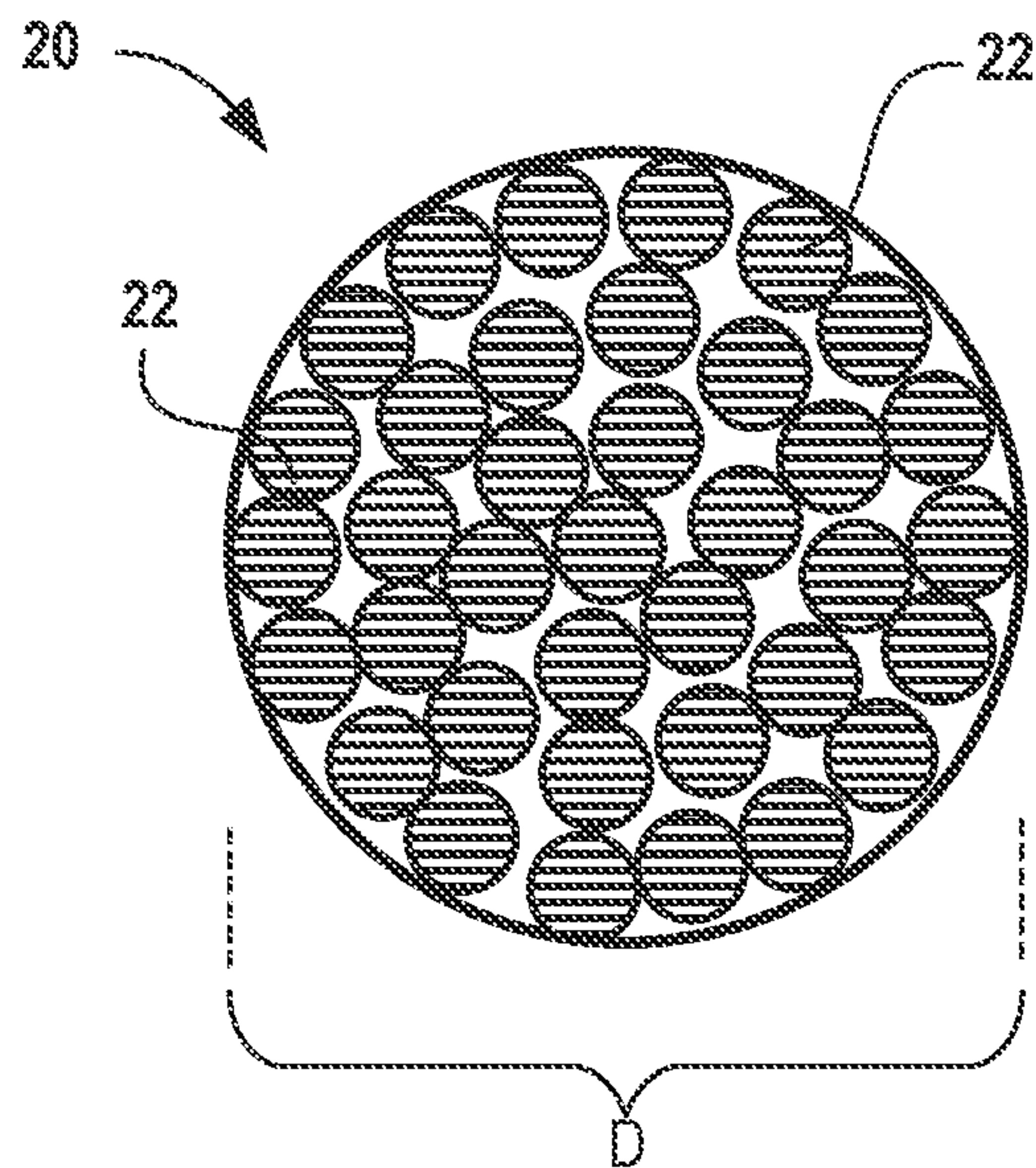


FIG. 1

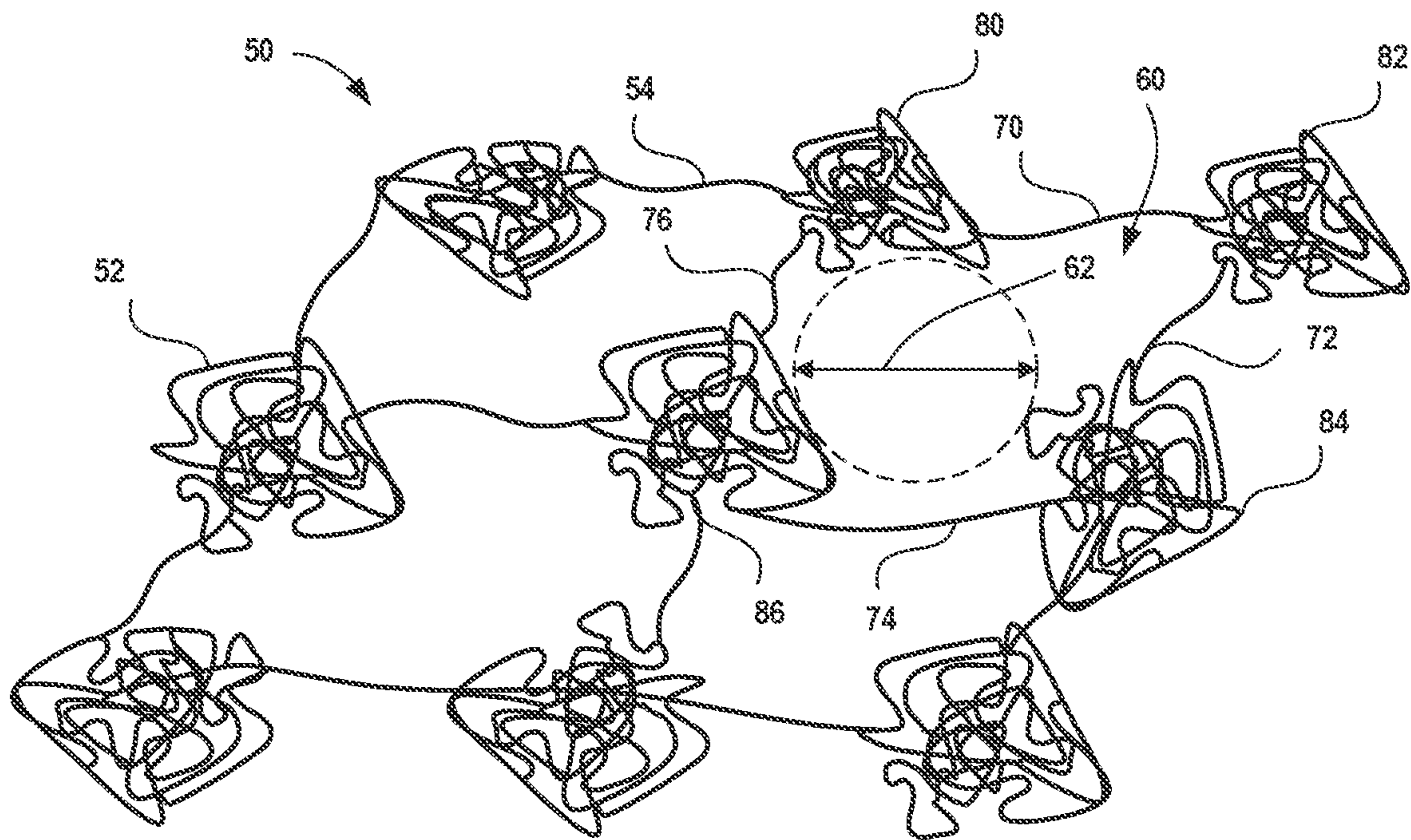


FIG. 2

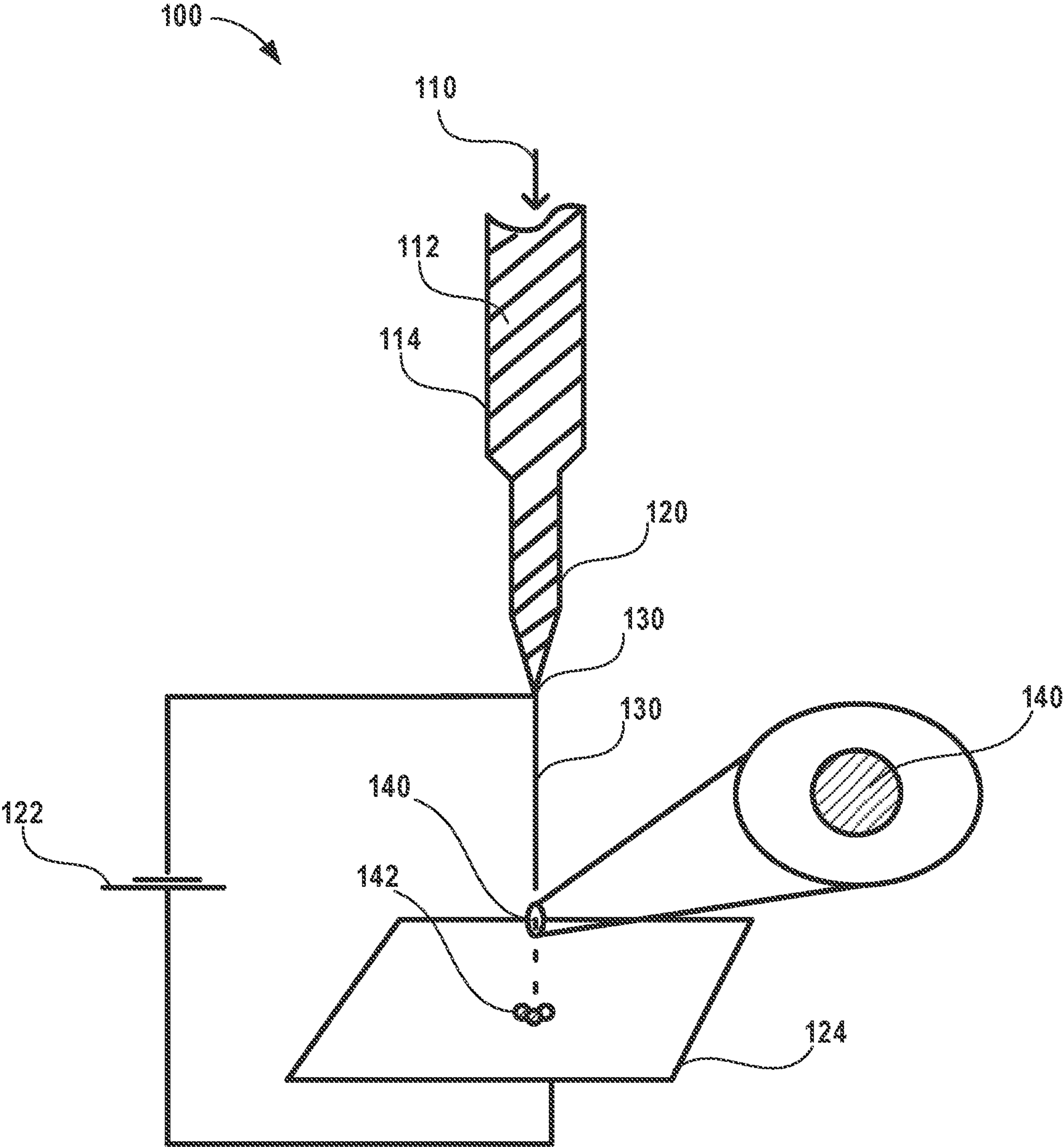


FIG. 3

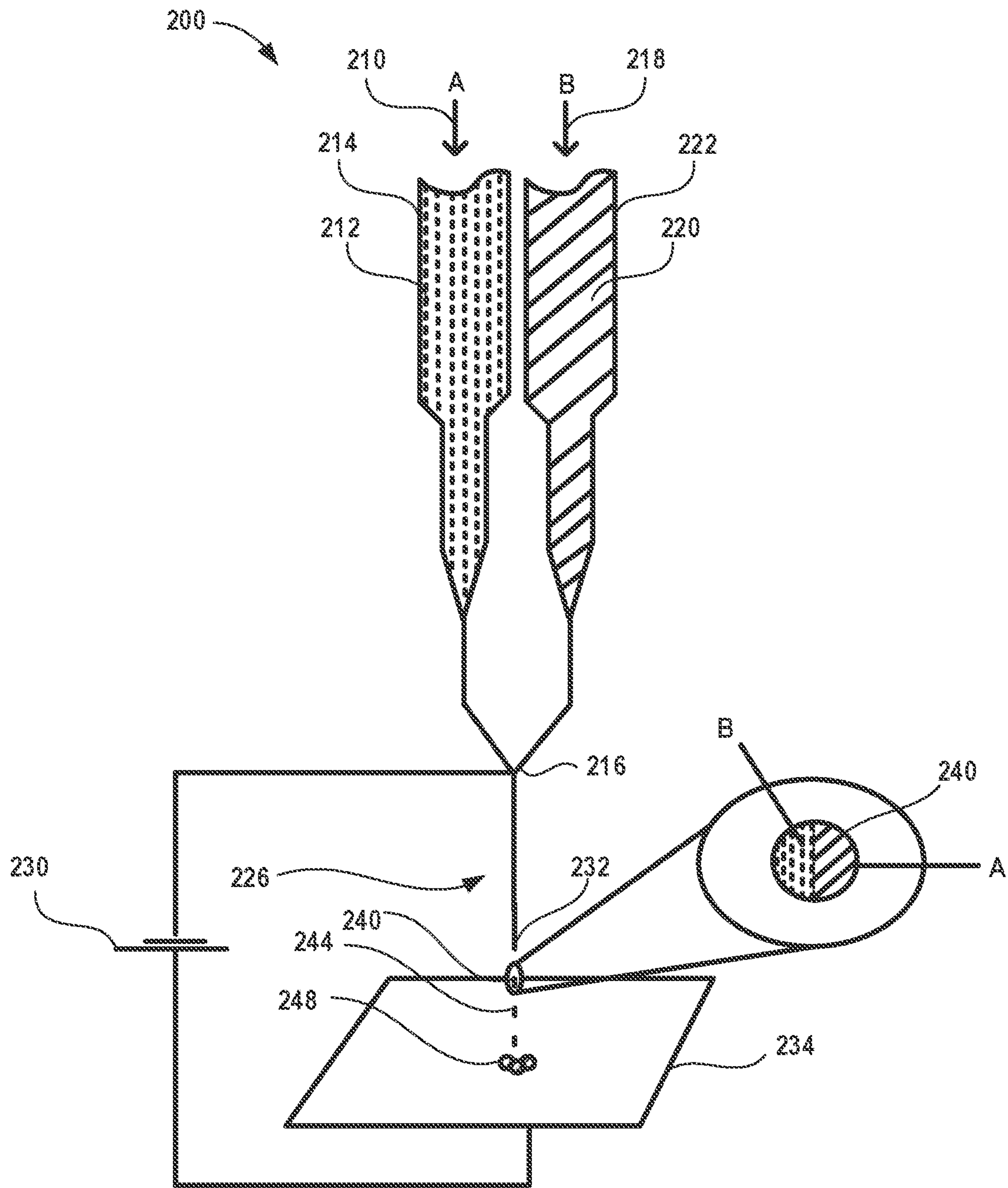
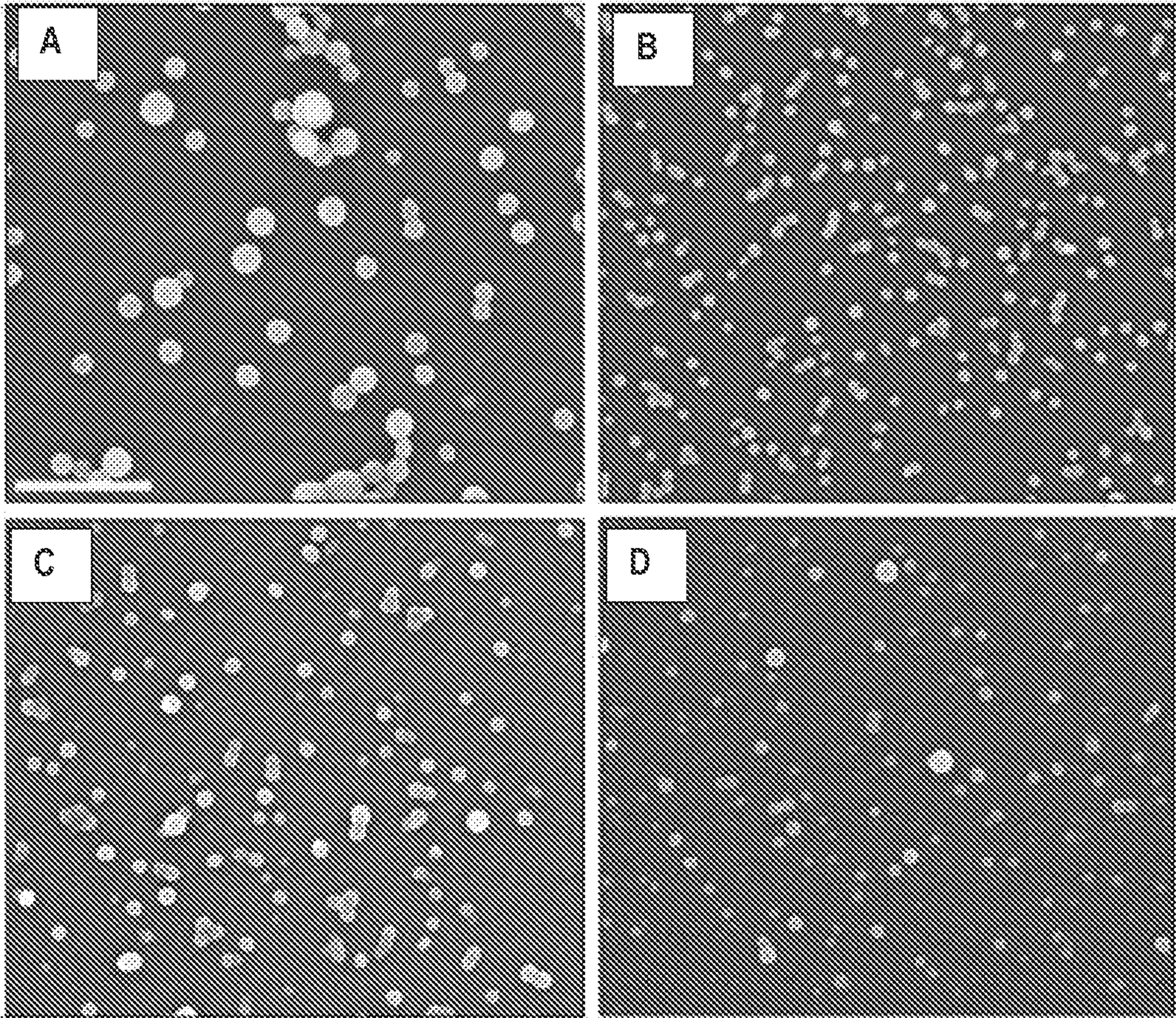
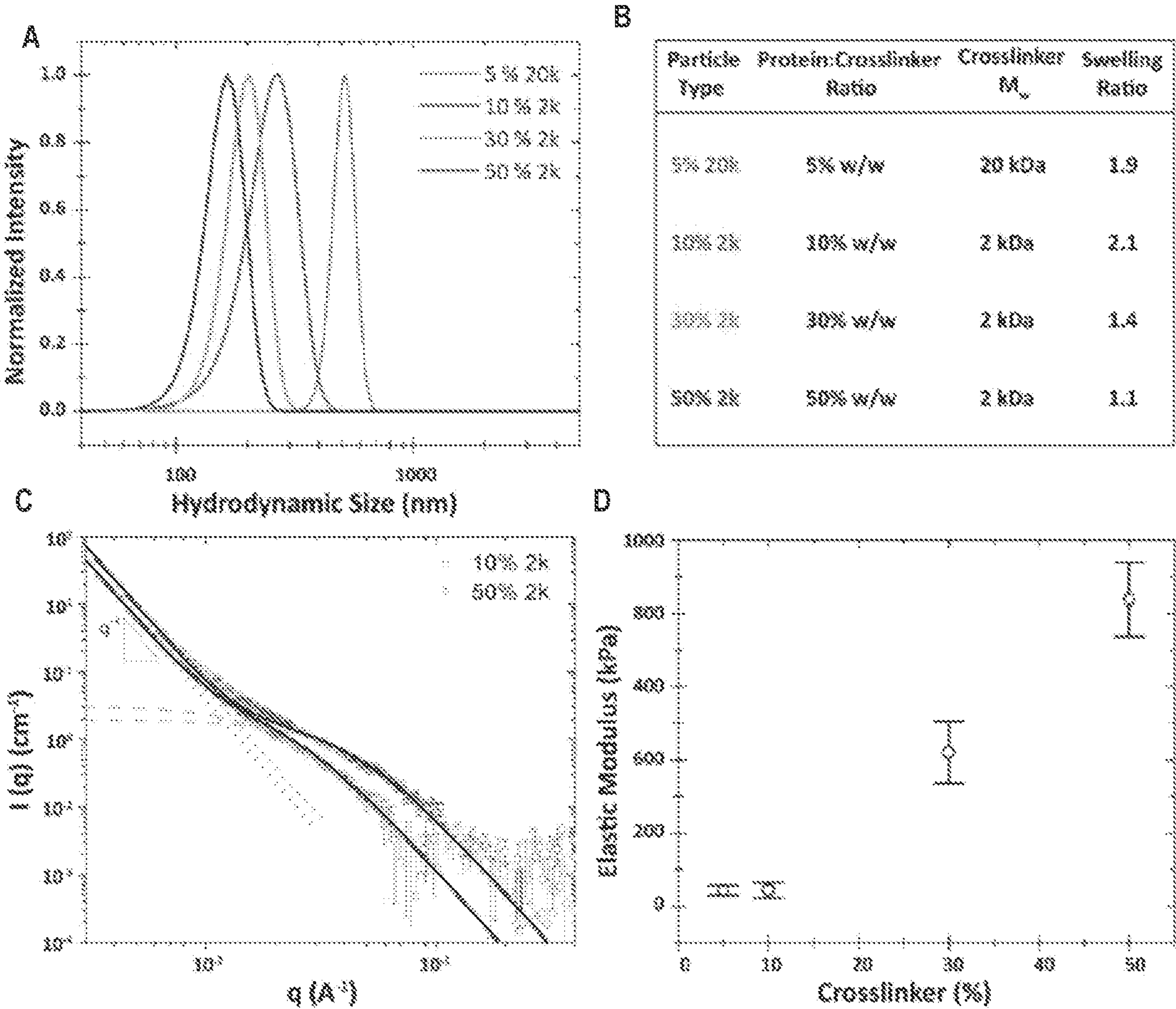


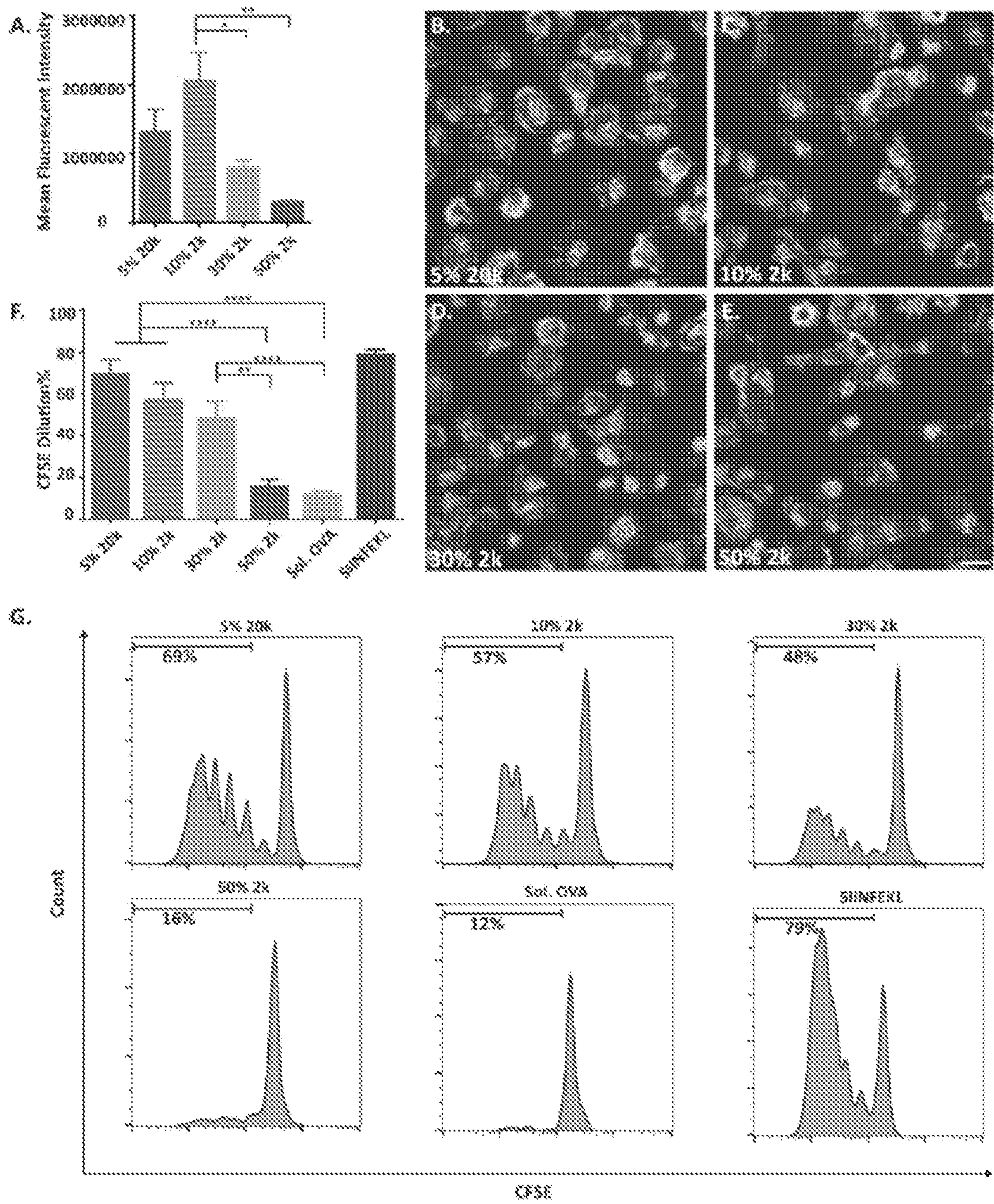
FIG. 4



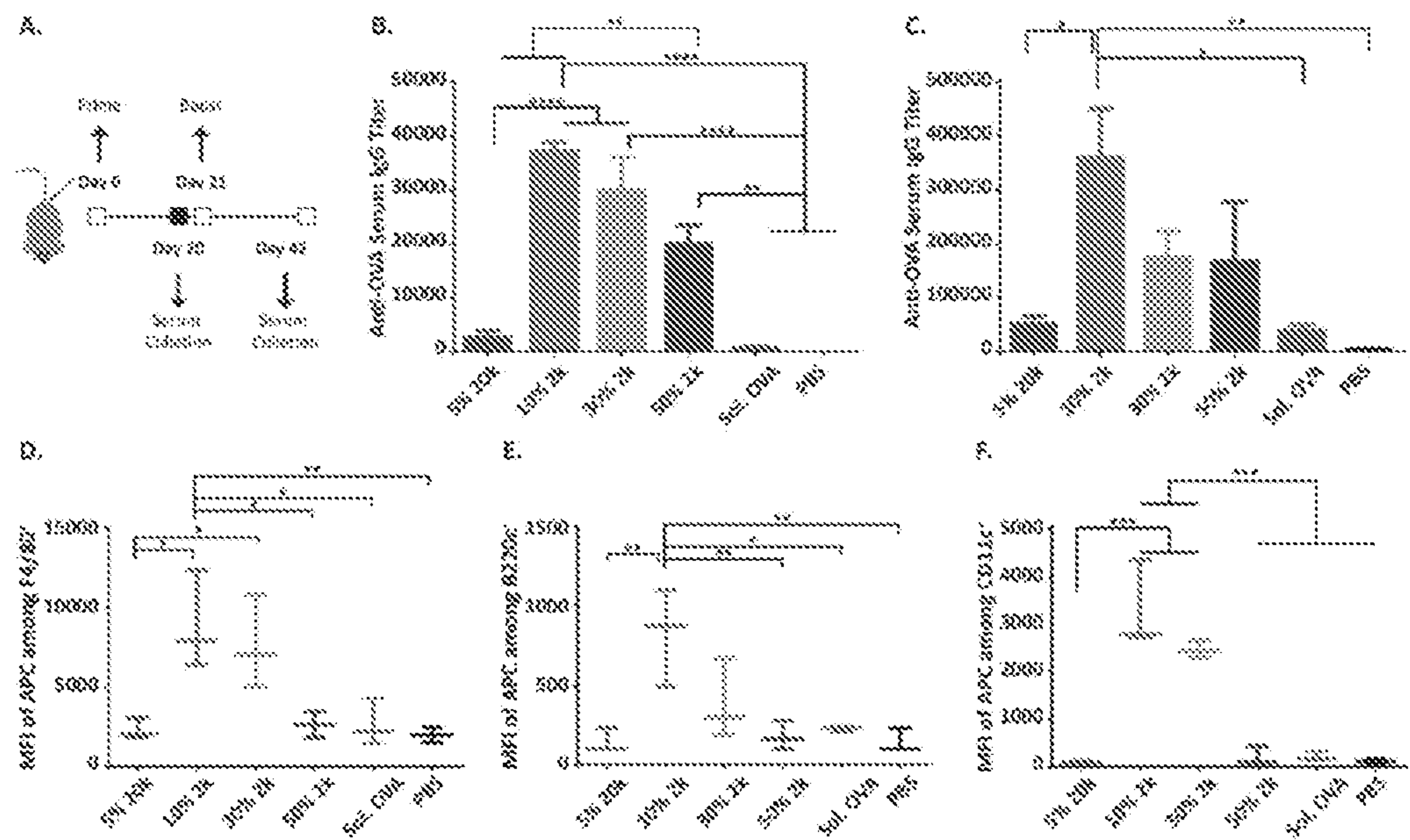
FIGS. 5A-5D



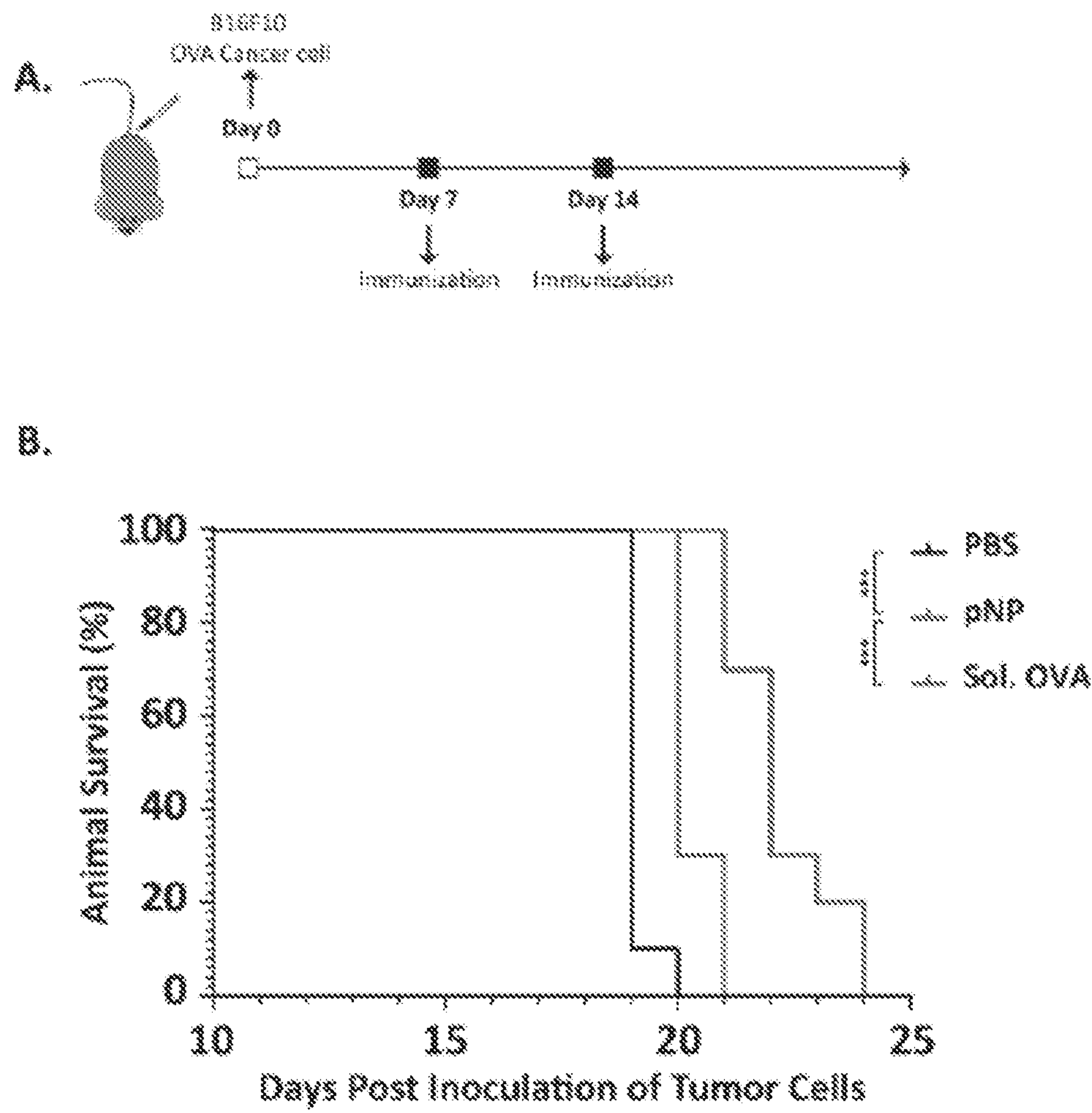
FIGS. 6A–6D



FIGS. 7A-7G



FIGS. 8A–8F



FIGS. 9A–9B

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2020/059497

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 9/00; A61K 9/14; B05D 1/04; B29C 67/00; C08J 3/00; C12N 5/00; C12N 5/02 (2021.01)

CPC - A61K 9/1694; A61K 9/5089; B82Y 5/00; B82Y 30/00; D01D 5/0069; D01D 5/32 (2021.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

see Search History document

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

see Search History document

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

see Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 2012/0045487 A1 (LAHANN et al) 23 February 2012 (23.02.2012) entire document	1-4, 14-17, 49-51, 57 ----- 22-25, 28
X -- Y	WO 2010/011641 A2 (THE REGENTS OF THE UNIVERSITY OF MICHIGAN et al) 28 January 2010 (28.01.2010) entire document	36-38 ----- 39
Y	US 2010/0233781 A1 (BANGERA et al) 16 September 2010 (16.09.2010) entire document	22-25, 28, 39
A	US 2013/0115169 A1 (LAHANN et al) 09 May 2013 (09.05.2013) entire document	1-4, 14-17, 22-25, 28, 36-39, 49-51, 57
P, A	HABIBI et al. "Engineered Ovalbumin Nanoparticles for Cancer Immunotherapy," Advanced Therapeutics, 09 July 2020 (09.07.2020), Vol. 3, Iss. 10, Pgs. 1-11. entire document	1-4, 14-17, 22-25, 28, 36-39, 49-51, 57

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

* Special categories of cited documents:

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

"D" document cited by the applicant in the international application

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

14 January 2021

Date of mailing of the international search report

09 FEB 2021

Name and mailing address of the ISA/US

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2020/059497

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 5-13, 18-21, 26, 27, 29-35, 40-48, 52-56, 58
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

- Remark on Protest
- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.