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Multifunctional Synthetic Protein Nanoparticles via Reactive Electrojetting

*Daniel F. Quevedo, Nahal Habibi, Jason V. Gregory, Yazmin Hernandez, Tyler D. Brown, Rikako Miki, Bradley N. Plummer, Sahar Rahmani, Jeffery E. Raymond, Samir Mitragotri, and Joerg Lahann**

Protein nanoparticles are a promising approach for nanotherapeutics, as proteins combine versatile chemical and biological function with controlled biodegradability. In this work, the development of an adaptable synthesis method is presented for synthetic protein nanoparticles (SPNPs) based on reactive electrojetting. In contrast to past work with electrohydrodynamic cojetting using inert polymers, the jetting solutions are comprised of proteins and chemically activated macromers, designed to react with each other during the processing step, to form insoluble nanogel particles. SPNPs made from a variety of different proteins, such as transferrin, insulin, or hemoglobin, are stable and uniform under physiological conditions and maintain monodisperse sizes of around 200 nm. SPNPs comprised of transferrin and a disulfide containing macromer are stimuli-responsive, and serve as markers of oxidative stress within HeLa cells. Beyond isotropic SPNPs, bicompartmental nanoparticles containing human serum albumin and transferrin in two distinct hemispheres are prepared via reactive electrojetting. This novel platform provides access to a novel class of versatile protein particles with nanoscale architectures that i) can be made from a variety of proteins and macromers, ii) have tunable biological responses, and iii) can be multicompartmental, a prerequisite for controlled release of multiple drugs.

D. F. Quevedo, N. Habibi, J. V. Gregory, Y. Hernandez, R. Miki, Dr. B. N. Plummer, Dr. S. Rahmani, Dr. J. E. Raymond, Prof. J. Lahann
Biointerfaces Institute
University of Michigan
Ann Arbor, MI 48109, USA
E-mail: lahann@umich.edu

D. F. Quevedo, Dr. B. N. Plummer, Dr. S. Rahmani, Dr. J. E. Raymond, Prof. J. Lahann, Dr. T. D. Brown, Prof. S. Mitragotri
Department of Biomedical Engineering
University of Michigan
Ann Arbor, MI 48109, USA

N. Habibi, J. V. Gregory Dr. B. N. Plummer, Dr. J. E. Raymond, Prof. J. Lahann
Department of Chemical Engineering
University of Michigan
Ann Arbor, MI 48109, USA

Dr. T. D. Brown, Prof. S. Mitragotri
Wyss Institute of Biologically Inspired Engineering and John A Paulson School of Engineering and Applied Sciences
Harvard University
Cambridge, MA 02138, USA

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/marc.202000425.

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Nanoparticle-based drug delivery systems provide improved drug stability, reduced drug toxicity, and improved biodistribution compared to free drugs.[1–3] There are a number of nanoparticle therapeutic platforms such as PEGylated liposomal doxorubicin (Doxil) and liposomal vincristine (Marqibo) that have been approved for cancer treatment.[4,5] Despite the progress made in the field of nanoparticle-based drug delivery, there are still unmet challenges such as poor circulation times, unwanted immunogenicity and a lack of adequate functional materials.[6,7] Solutions to these challenges often conflict with each other, leading to the development of multifunctional, multicompartmental nanoparticles.[8] Multifunctional nanoparticles have traditionally been made of synthetic polymers, such as polyethylene glycol (PEG),[9] poly(lactide-*co*-glycolide) (PLGA),[10] or block copolymer systems.[11] Using proteins as the primary building block of nanocarriers could be an appealing alternative due to their chemical diversity, inherent biological functions, and a potentially reduced risk for immunogenicity.[12]

Protein nanoparticles (PNPs)[6,12] have been pursued for drug delivery applications including the clinically approved drug Abraxane[13] and other preclinically studied candidates.[14–16] Common PNP fabrication methods include, among others, nab technologies,[17,18] coacervation,[19,20] and self-assembly.[21,22] Despite undoubtable progress in recent years, PNP technologies are still hampered by a range of drawbacks. While PNPs prepared via nab technologies have been implicated with decreased morbidity,[23] the processing conditions during particle preparation have been showed to cause protein denaturation.[24] Coacervation can create large quantities of PNPs,[25] but generally lacks sufficient control to prepare multifunctional nanoparticles. Self-assembly can provide more structural diversity[26] but requires ab initio design of new protein building blocks that has to be done separately for each application. Except for the more involved self-assembly route, none of these techniques has so far resulted in architecturally controlled protein nanocarriers, such as bi- or multicompartmental nanoparticles.

Electrohydrodynamic (EHD) cojetting has previously been shown to be effective at creating multicompartmental particles with nanoscale anisotropy.[8] EHD cojetting relies on laminar coflow of two or more polymer solutions prior to the jet ejection to pretemplate compartmentalized nanoparticles and nanofibers, with fine control over size, shape, composition, and spatial distribution of matter at the surface and bulk level.[27,28] EHD cojetting has been used to fabricate multicompartmental polymer particles that incorporate various functionalities, such as stealth modalities,[29] targeting/tracing,[30] and encapsulation of different cargos such as siRNA,[31] imaging agents,[32] and small molecule cancer drugs.[33]

As traditional protein nanoparticle synthesis methods lack control over anisotropy, we have developed reactive electrojetting as a method for making anisotropic synthetic protein nanoparticles (SPNPs). Reactive electrojetting takes advantage of the anisotropic control afforded by EHD cojetting to create protein nanoparticles, and then introduces a second chemical step that converts the particles into nanogels through a sol–gel transition using a variety of macromers.

To prepare SPNPs using reactive electrojetting, particles are first made using EHD jetting. Protein is dissolved in an aqueous solvent system with 10% ethanol. The addition of an organic solvent increases the volatility and decreases the surface tension of the solution.[34] Solid nanoparticles are then prepared by accelerating the jetting solution in an electrical field created between the tip of the jetting needle and a collection plate (**Figure 1**a). Once the electrical potential is applied, a Taylor cone is spontaneously formed[35] and the jet is ejected from the Taylor cone toward the collection plate. In EHD jetting, conditions (i.e., surface tension, flow rate, solute concentration, and applied electric field) can be controlled to result in either particle or fibral formation. The protein concentrations in all jetting solutions were maintained 10% w/v or lower to ensure that only particles were formed. After EHD jetting, particles were examined for uniformity using scanning electron microscopy (SEM) (Figure 1b, Figures S3-S5, Supplemental Materials). Circular dichroism spectroscopy demonstrated that component proteins had unchanged secondary structures compared to their native confirmations (Figure S2, Supporting Information).

The second step of reactive electrojetting is the reaction of the proteins in the nanoparticles with a variety of reactive macromers, such as short *N*-hydroxysuccinimide (NHS)-ester functionalized PEG chains (Figure 1c). The reaction occurs during or immediately after the EHD jetting process, rendering the SPNPs stable in aqueous environments and locking in their geometry. A small library of commercially available macromers was selected to investigate different sol/gel transitions. The first two macromers, 2 KDa O,O′-bis[2-(*N*-succinimidyl-succinylamino)ethyl]polyethylene glycol (PEG-NHS) and 4,7,10,13,16,19,22,25,32,35,38,41,44,47,50,53-hexadecaoxa-28,29-dithiahexapentacontanedioic acid di-*N*-succinimidyl ester (PEG-NHS-S), react the macromers' ester functional groups with the proteins' amine groups. This reaction completes after SPNPs are deposited by EHD jetting onto the collecting surface and then placed at 37 °C for 7 days. The third macromer, glutaraldehyde (GA), binds proteins together by reacting aldehyde groups with a variety of protein residues.[36] GA crosslinking is conducted immediately after EHD jetting, when dried protein particles are placed in a sealed container containing 20% glutaraldehyde, which vaporizes and reacts at room temperature. The last crosslinking method does not rely on a macromer but instead takes advantage of native disulfide bonds within proteins (S-S). Prior to EHD jetting, proteins are treated with trifluoroethanol (TFE) and β-mercaptoethanol (BME) to disrupt

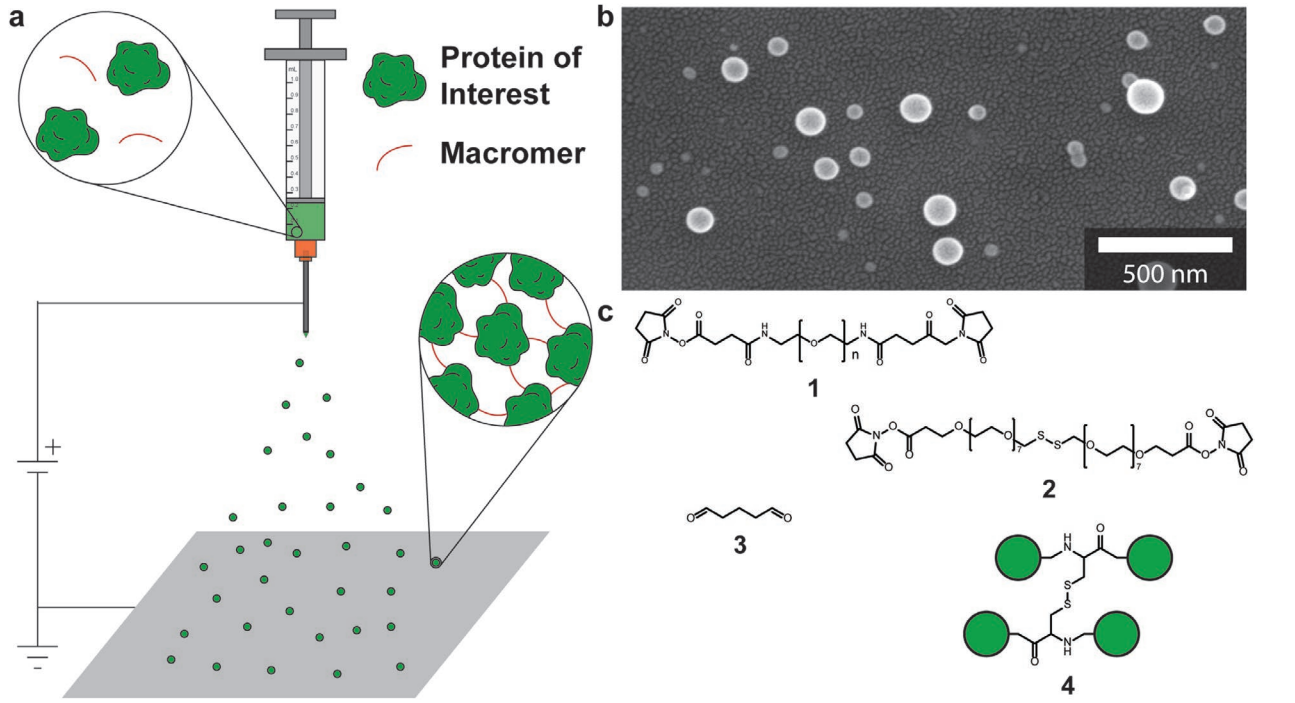

Figure 1. a) Preparation of synthetic protein nanoparticles (SPNPs) using reactive electrojetting. b) SEM images of particles made using EHD jetting. Particles are jetted, and subsequently c) polymerized using a variety of different macromers: 1. (PEG-NHS), 2. (PEG-NHS-S), 3. (GA), and 4. (S-S)

native protein structure and break intermolecular disulfide bonds.[37,38] The solution is then jetted as described previously. While droplets are traveling to the collecting surface, TFE and BME evaporate allowing the disulfide bonds to reform between proteins, resulting in insoluble SPNPs on the collecting surface.

PEG-NHS was selected as a biocompatible and biodegradable macromer that can be imparted with functional groups. PEG-NHS-S showcases the flexibility of macromers based on PEG-NHS. By incorporating stimuli responsive groups into the PEG chain, such as the disulfide in the PEG-NHS-S, particles can be made to react in response to different environments. GA vapor treatment was developed as a faster alternative to PEG-NHS macromers, as the vapor-phase reaction occurs in as little as 30 min to form fully water insoluble SPNPs. Thus, GA allows for the incorporation of time sensitive agents into SPNPs, such as radiotherapeutics. S-S was developed to have a method which does not use any available functional groups on the protein residues, unlike the three other macromers. Additionally, S-S takes place in an organic solvent system, as opposed to the aqueous system used for the other macromers. This different solvent system introduces the ability for SPNPs to be loaded with hydrophobic drugs, opening up a large number of potential therapeutics for drug delivery with SPNPs.

After the reactive electrojetting process, the resulting particles have typical diameters at or below 100 nm and are monodisperse (PDIs range from 0.15 to 0.23), as seen by SEM (Figures S3–S5, Table S2, Supporting Information). To further narrow particle size distribution, particles were first collected, then sonicated to cause disaggregation, and were size purified using a previously established serial centrifugation technique.[32] After hydration, particles made with human transferrin (hTf) and each of the different macromers were measured using dynamic and electrophoretic light scattering (DLS and ELS) and were found to have similar size distributions and zeta potentials (Figure S6, Supporting Information).

To show how macromer conditions can be used to tailor SPNPs hydrodynamic size, human serum albumin (HSA) particles were made with PEG-NHS. The hydrodynamic size was tuned by changing the macromer to protein ratio. Increasing the ratio of macromer to protein does not overtly appear to alter the diameter of the particles in the dry state relative to increasing ratio. However, as the amount of macromer is increased, the SPNPs expand less under hydrodynamic conditions. This can be seen observed as a 33% decrease in the mean hydrodynamic diameter as one goes through the series from 10% to 40% macromer (Figure S7–S8 and Table S2, Supporting Information). It should be noted that these measurements were of centrifuged particles in their hydrated state, in order to allow for an accurate comparison to particles in their dry state.

We also explored how reactive electrojetting can make SPNPs from a variety of proteins. A small library of proteins was selected to synthesize SPNPs, each with potential biomedical applications: Human transferrin,[39] insulin (Ins),[40] hemoglobin (Hem),[41] and lysozyme (Lys).[42] For these experiments, each protein was used to fabricate stable SPNPs with PEG-NHS. Proteins were dissolved as previously described, with the exception of Ins which was dissolved by adding 10% acetic acid to the solvent mixture, due to poor solubility in neutral aqueous conditions. SEM images of SPNPs as sampled from the collecting surface demonstrated that different proteins did not affect the morphology of the resulting particles (**Figure 2**a–d). To evaluate

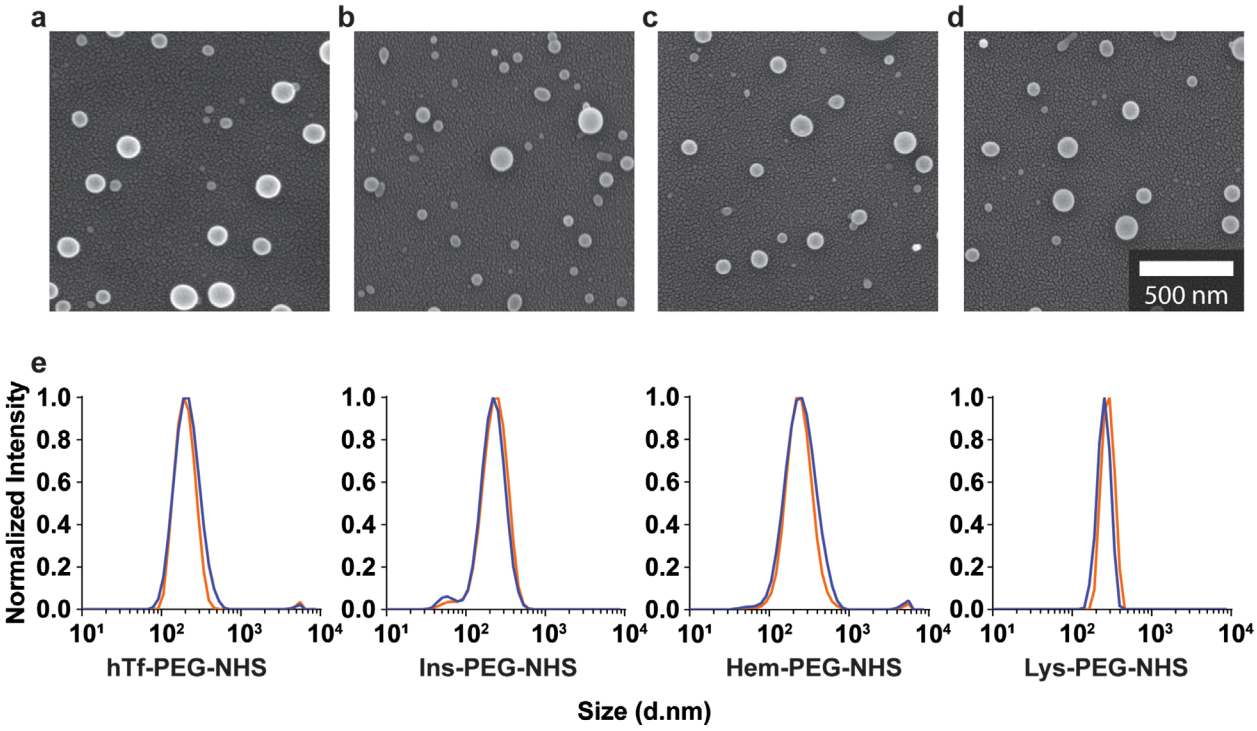

Figure 2. Stable synthetic PNPs can be made using a variety of proteins. SEM images of SPNPs made from a) hTf-PEG-NHS, b) Ins-PEG-NHS, c) Hem-PEG-NHS, and d) Lys-PEG-NHS. e) The stability of particles in phosphate buffered saline (PBS) over a 1 week period was characterized by measuring the particles using DLS 1 d (blue trace) and 7 d (red trace) after synthesis and size purification.

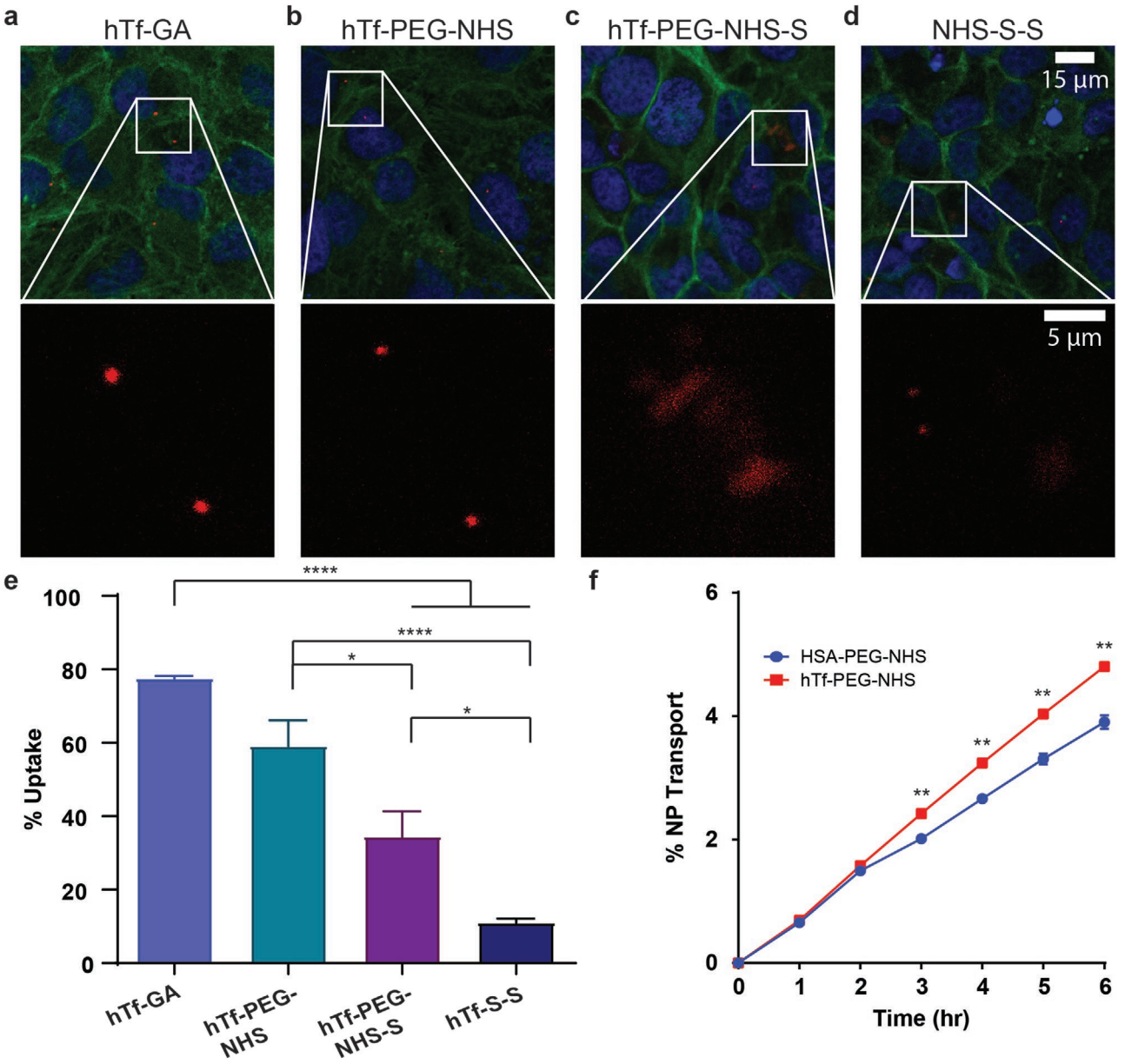

Figure 3. SPNPs can be made using different methods that have a distinct effect on the in vitro uptake and behavior of the particles. a–d) Fluorescent SPNPs made with different macromers were added to HeLa cells for 1 h and their behavior studied using confocal microscopy. e) Uptake was quantified using confocal microscopy using HeLa cells cultured at equivalent conditions and with SPNPs added for 24 h (One-way analysis of variance (ANOVA), followed by Tukey's post-test). f) SPNP BBB transport. Percentage transport of HSA and hTF SPNPs across hCMEC/D3 monolayers in Transwell inserts (nonpaired, two-tailed *t*-test). (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$). Values are reported as mean ± SEM (standard error of the mean).

the stability of the particles, SPNPs were collected, size purified, and stored at 4 °C for 7 days. Their size distributions after a week showed no significant difference, confirming particle stability after storage (Figure 2e and Table S2, Supporting Information).

Various experiments were conducted to explore how protein and macromer choice affects SPNP behavior in biological systems. To investigate the effects of different macromers on the in vitro behavior of SPNPs, hTf SPNPs were synthesized with each of the different macromers and loaded with fluorescently labeled bovine serum albumin. SPNPs were then incubated with HeLa cells. SPNPs behaved differently depending on their macromer. hTf-PEG-NHS and hTf-GA SPNPs remained punctate when observed using confocal microscopy (**Figure 3**a,b). In contrast, hTf-PEG-NHS-S and hTf-S-S SPNPs, which rely on disulfide-bonds for structure, were more diffuse (Figure 3c,d). It is likely that these particles degraded due to disulfide bonds breaking in the cellular redox/reducing environment.[43] These effects have been observed in other particles made with similar chemical principles.[19]

The uptake of SPNPs was evaluated quantitatively by flow cytometry (Figure 3e). There was an observed difference in the

uptake level for SPNPs based on macromer. Cells incubated with hTf-GA showed a sevenfold greater uptake percentage than those exposed to hTf-S-S ($P < 0.0001$), and a twofold increase compared with hTf-S-S ($P < 0.0001$). Uptake values for cells incubated with hTf-GA and hTf-PEG-NHS were not statistically different. As the particles made using different macromers had no significant differences in size distributions or zeta potentials, the reasons for these observed differences are yet unknown. We hypothesize that these effects may be caused by differences in the secondary structures of the proteins after they undergo polymerization following jetting, or in the mechanical properties of the SPNPs, and this will be explored in future studies.

To evaluate the potential of SPNPs' variable protein composition, we compared the blood brain barrier (BBB) permeability of hTf-PEG-NHS SPNPs to human serum albumin SPNPs (HSA-PEG-NHS), which have been previously developed.[44] A static in vitro BBB model was constructed using a Transwell migration assay which analyzes cellular transport across an analogue of the BBB, where hCMEC and D3 cells are placed in the apical compartment.[45] SPNPs were fluorescently tagged, and particles that were able to go from the apical to the basolateral compartments of the assay were measured using fluorometry. hTF-PEG-NHS SPNPs exhibited higher percentage transport across the BBB model ($P < 0.01$), compared to HSA-PEG-NHS SPNPs (Figure 3f) as expected, due to the overexpression of transferrin receptors on brain endothelium. This proof-of-concept study shows potential for these SPNPs to be explored for BBB targeting in future studies.

Finally, we sought to demonstrate how reactive electrojetting can synthesize anisotropic SPNPs (ASPNPs). Cojetting is a well-established method where a parallel capillary system is used to create laminar coflow in EHD jetting.[27] To demonstrate that this technology can be applied to SPNPs, particles were made that had one compartment containing fluorescent bovine serum albumin (BSA) and the other fluorescent hTf. These particles were processed as previously described and then imaged using structured illumination microscopy (SIM). As can be seen in **Figure 4**, the resulting particles are clearly composed of two separate compartments that are easily resolved.

In this work, we have developed a method for the synthesis of synthetic protein nanoparticles fabricated using reactive electrojetting with tunable material compositions. Anisotropic SPNPs were also developed. As each compartment can be individually designed, this technique allows for the development of complex nanoparticles such that release kinetics of drug cargo from each compartment could be independently controlled, as shown in previous studies.[46,47] Novel nanoparticle-based cancer vaccines, where each compartment is made of a different cancer antigen, could provide significant therapeutic advantages. Additionally, the use of functional proteins could also lead to the delivery of protein antigen,[48] active gene therapy enzymes and nucleic acids. Recent work has shown how a treatment based on SPNPs cured mice in an intracranial murine glioblastoma model and prevented subsequent tumor recurrence from a secondary implant, suggesting immunity to cancer recurrence.[44] SPNPs have thus already started to demonstrate their potential in both the therapeutic and preventive clinical spaces. In the

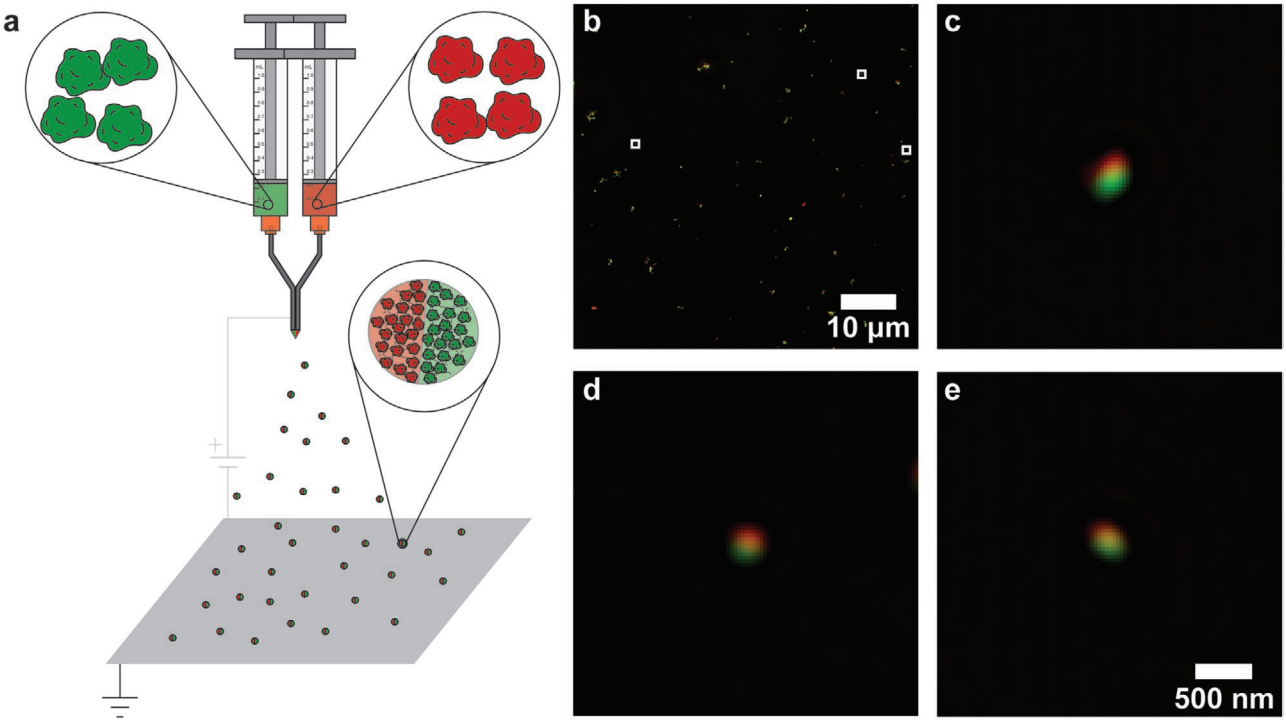

Figure 4. EHD cojetting can be used to make bicompartmental SPNPs. a) SPNPs containing human serum albumin (HSA) in one compartment and human serum transferrin (hTf) in the other were synthesized, with each compartment doped with BSA-Alexa-488 or hTf-Alexa-647, respectively. The particles were imaged using SIM. b) The images were then deconvoluted. c–e) Zoomed in images of individual particles are shown to showcase the bicompartmental nature of the particles.

future, we aim to further develop these ASPNPs into a variety of clinical applications.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

anisotropy, drug delivery, multifunctionality, nanomedicine, synthetic protein nanoparticles

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