CREATION OF BIFUNCTIONAL PARTICLES WITH SPATIALLY SEGREGATED PROTEINS

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CREATION OF BIFUNCTIONAL PARTICLES WITH SPATIALLY SEGREGATED PROTEINS

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To my parents, Thanes and Elenore Tang

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LIST OF ABBREVIATIONS

AFM	Atomic force microscope
APTES	(3-Aminopropyl)triethoxysilane
BSA	Bovine serum albumin
EDAC	1-Ethyl-3(dimethylaminopropyl)carbodiimide
FITC	Fluorescein isothiocyanate
FN	Fibronectin
G	Gravity
IgG	Immunoglobulin G
SA	Streptavidin
TRITC	Tetramethyl rhodamine iso-thiocyanate

SUMMARY

We present a fabrication process to create bifunctional microparticles displaying two different proteins have been spatially segregated onto hemispheres. Silica and polystyrene microparticles with 2.0 μm, 4.08 μm, and 4.74 μm diameters are processed with metal deposition to form two chemically distinct and segregated hemispheres. The surface of each hemisphere is then separately derivatized with proteins using different chemical conjugation strategies. These bifunctional Janus particles possess biologically relevant, native conformation proteins attached to a biologically-unreactive and safe substrate. They also display high densities of two types of spatially segregated proteins which may enable a range of capabilities that monofunctional particles cannot, such as improved targeting of drug carriers and bioimaging agents.

CHAPTER 1

INTRODUCTION

1.1 Janus Particles

In 1991, De Gennes first coined the term "Janus" in his Nobel lecture to describe particles with chemically distinct hemispheres after the two-faced Roman god, Janus.[1,2] Chemically unique hemispheres can enable multifunctional capabilities which can be used in a wide range of applications. Janus particles have been integrated with other existing systems to create stabilizing emulsions,[3,1] electronic paper,[1,2,4,5] imaging,[6] sensors,[4,7] and drug delivery systems.[8,1]

Many researchers have been developing new and innovative fabrication methods for Janus particles.[1,7,9,10,3,8,11] Chemical Janus particles and Janus particles with only one side chemically altered have been successfully fabricated using several methods, including microfluidics,[3,5,7,12] dual supplied spinning disks,[1] and selective surface modifications.[11,13,5,8,14] Selective surface modifications can be created through microfabrication techniques such as evaporation[15] and sputtering[4,10] in which a hemisphere is temporarily or permanently masked such that the exposed hemisphere can be selectively activated.[3,1,13,5,8,14]

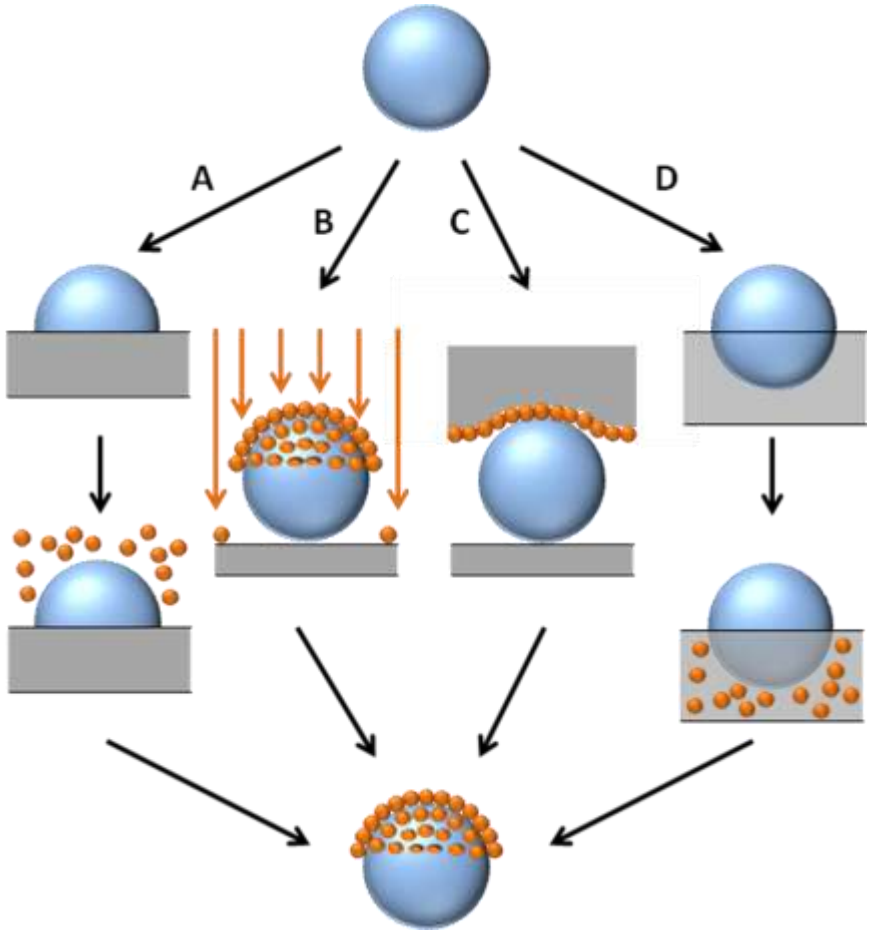


Figure 1.1. Common techniques for surface modifications. (A) Masking/unmasking, (B) directional fluxes or fields, (C) microcontact printing and (D) partial contact with a reactive medium. Adapted and redrawn from ref. 1.

In 2004, Paunov developed a novel fabrication method, called the gel trapping technique, to create anisotropic Janus particles using the Langmuir-blodgett trough in which particles assemble at liquid-liquid interfaces to be functionalized.[10] In this method, an even monolayer of polystyrene particles are suspended at the water-oil or decane-water interface at 50ºC. A gelling agent, Gellan, is mixed with the hot water prior to the particle suspension and allows the water to set as the liquids cool to 25ºC. The particles are held in place in the gelled water as the oil or decane is removed and replaced with the polymer poly(dimethylsiloxane) (PDMS). PDMS is then cured and peeled away from the gelled water. The polystyrene particles are lifted onto the PDMS and can be removed

from the polymer through the localized mechanical stretching of the pores. To create Janus particles with two chemically unique surfaces, Paunov utilized a gold sputtering technique prior to the particle removal. However, after the gold sputtering process, the particles were unable to be removed via the localized stretching of PDMS and were thus mechanically removed with a blade. This particle removal method has the potential to damage particles and their gold surfaces, and is therefore an unreliable fabrication process to create two consistently distinct surfaces for bifunctional Janus particles. For our study, a consistent coverage of gold was needed in order to spatially segregate the proteins evenly, so this method was inadequate to our needs.

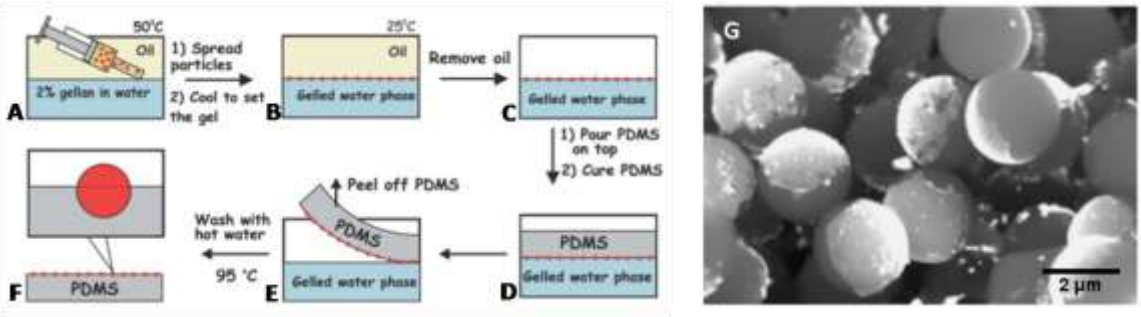


Figure 1.2. Schematic of gel trapping technique for replicating microparticle monolayers at the oil-water (A-F) and air-water (C-F) interfaces. (G) Scanning electron microscopy (SEM) image of Janus particles fabricated by gold sputtering followed by a mechanical removal of particles from the PDMS.[10]

Microfluidic principles can also be applied to create Janus particles.[7] Nie created Janus particles, ranging from 40 to 60 µm in diameter by flowing two miscible monomers or polymers in parallel, and are then mixed as they pass through a sharp interface. Janus particle droplets form and are quickly polymerized with a UV source. The size of these Janus particles depends on the sharpness of the interface and the fluid flow. After this fabrication process, fluorescently labeled BSA was conjugated onto the particle hemisphere, creating half-coated particles with a single protein conjugated to one hemisphere. However, this fabrication method does not spatially segregate two proteins

and small sizes are important for specific biological processes, such as enhanced permeability retention. The larger particles may also potentially clog blood vessels and are relatively large compared to existing drug delivery carriers, so this Janus particle fabrication method undesirable for many *in vivo* applications.

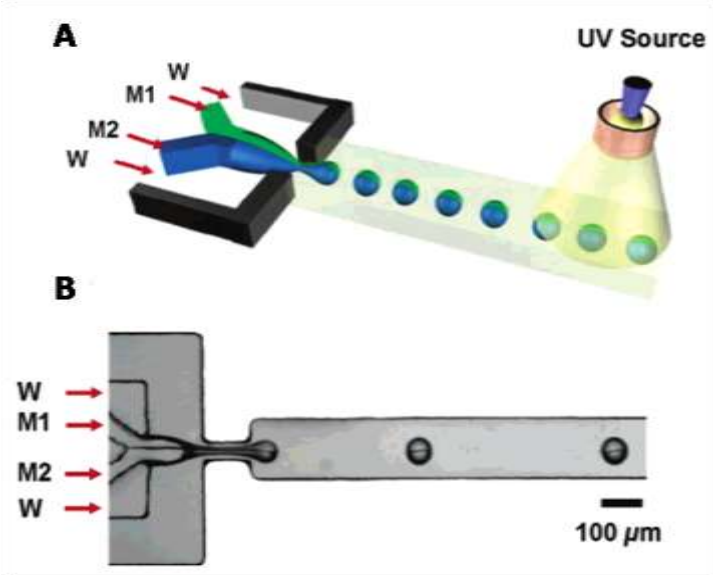


Figure 1.3. Schematic of generation of Janus droplets via microfluidics. (A) Generation of Janus droplets from immiscible monomers M1 and M2, emulsified in an aqueous solution of sodium dodecylsulfate (W). The droplets are irradiated with UV light in the downstream channel. (B) Optical microscopy image of formation of Janus droplets. FITC conjugated bovine serum albumin was later conjugated to one of the polymers, coating one hemisphere on the Janus particle.[7]

Other studies used microcontact printing to stamp particles with a variety of inks that can create fluorescent, protein-binding, or magnetic Janus particles. [9] Yet this is a time-intensive process and results in stamped particles that are not fully coated in the inks. Therefore these particles possess a gap between the two printed areas of contact, as can be seen in Figure 1.4.

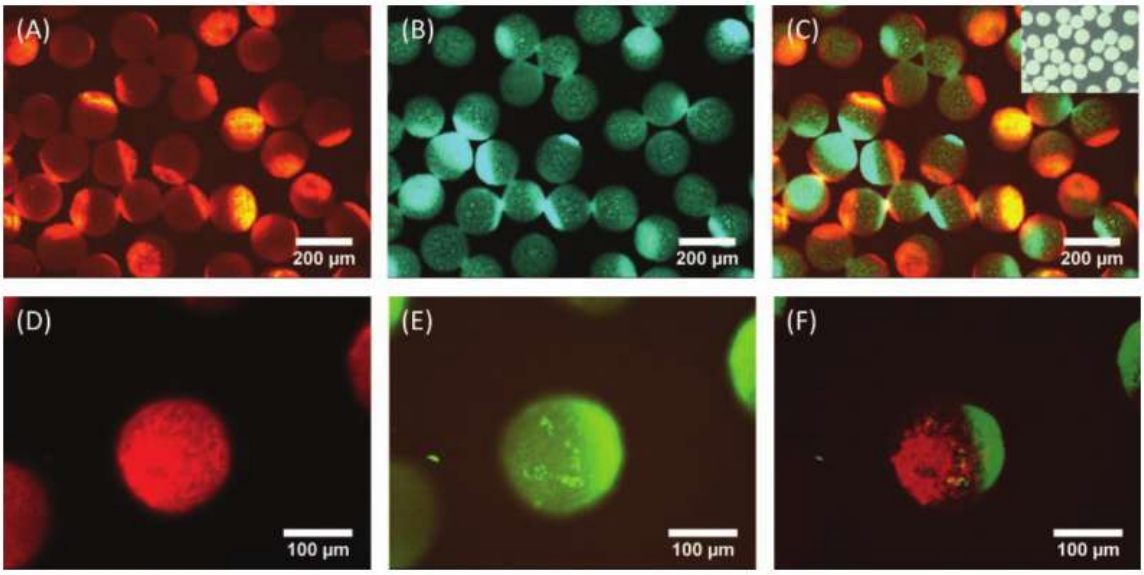


Figure 1.4. Janus particles obtained via microcontact printing. Upper row: Janus polymer particles obtained by printing red and blue dyes. (A) Rhodamine ethylenediamine (green filter); (B) dansylcadaverine (UV filter); and (C) overlay of both images. Inset: bright field image. Bottom row: Janus polymer particles obtained by printing carbohydrates: (D) rhodamine-labeled peanut agglutinin bound to β-galactoside-modified faces (green filter); (E) fluorescein labeled Con A bound to α-mannoside faces (blue filter): and (F) overlay of both images.[9]

1.2 Motivation

Janus particles, or particles with two chemically unique hemispheres, are of interest for their potential in a wide range of applications,[1,9,2] such as stabilizing emulsions,[3,1] electronic paper,[1,2,4,5] imaging,[6] sensors,[4,7] and drug delivery systems.[8,1] The two distinct hemispheres have the potential to create a multifunctional capability that can spatially link two processes that are difficult to artificially couple in a single molecule or particle otherwise, such as specific recognition of ligands and activation of metabolic processes. In these drug delivery systems, bifunctional Janus particles can potentially be utilized as advanced pharmaceutical agents to enhance targeting of tissues and cells through ligands on one hemisphere while simultaneously stimulating biological pathways with activation moieties from the second hemisphere.

While there is a great amount of research on carriers for drug delivery,[12,16–18] drug targeting would be an important improvement in the drug development and delivery process. Janus particles have a role to play for improved drug delivery by imparting multifunctional capabilities via protein conjugations.[8,13] Other research studies have created Janus particles with a single protein type conjugated to one hemisphere.[7,19] However, there are currently no known fabrication methods to produce bifunctional Janus particles. Bifunctional Janus particles are a challenge to fabricate as the synthesis technique requires consideration of protein functionality and stability.[20] Monofunctional and spatially uniform microparticles can be routinely produced through the surface modification of particles. These methods result in a nominally uniform distribution of proteins that creates monofunctional particles with a single targeting or stimulating capability.[21,16,17] However, the creation of bifunctional particles may enable a range of capabilities that monofunctional particles are unable to achieve, such as improved targeting of drugs or advanced bioimaging agents.

1.3 Objectives

Bifunctional particles are a challenge to fabricate as the synthesis technique requires consideration of protein functionality and stability.[20] The goal of this study was to successfully fabricating bifunctional particles, or particles that display spatially segregated proteins, in large quantities. To achieve this goal, the fabrication processes must address protein stability in the pH and temperature range, and solvent concentrations necessary to the fabrication process.

CHAPTER 2

METHODS

Currently there are no described methods to produce Janus particles with two types of separated proteins. In this study, several procedures have been developed to modify uniform microparticles with a metal coating on one hemisphere and then to conjugate different types of spatially segregated proteins onto the surfaces using different chemistries for the two hemispheres. The microparticles were modified by depositing a gold film on one side such that each particle displays a conjugation moiety on each hemisphere: a gold surface and the original surface consisting of biotinylated polystyrene, carboxylated polystyrene, or silica. These particles were commercially available and were used as a platform shape that was later modified to contain reactive groups.

1-Ethyl-3(dimethylaminopropyl)carbodiimide (EDAC), PolyLink coupling buffer solution, silica particles (diameter = 4.74 μm), and two sizes of carboxyl polystyrene particles (diameter = 2.122 μm and 4.08 μm) were purchased from Bangs Laboratories Inc. (Fishers, IN). Biotin-conjugated polystyrene microparticles with 2 μm diameters were purchased from Polyciences (Warrington, PA). (3-Aminopropyl)triethoxysilane (APTES), rabbit immunoglobulin G (rIgG) antibody, fluorescein isothiocyanate (FITC) conjugated bovine serum albumin (BSA), and unlabeled BSA were purchased from Sigma-Aldrich (St. Louis, MO). Alexa Fluor 488 protein labeling kit, Alexa Fluor 488 conjugated goat anti-rabbit IgG, Alexa Fluor 647 conjugated with streptavidin (SA), tetramethylrhodamine-6-isothiocyanate (TRITC), phosphate-buffered saline (PBS), and natural human fibronectin (FN) were purchased from Invitrogen (Carlsbad, CA). 8-Amino-1-octanethiol was purchased from Dojindo Molecular Technologies Inc. (Rockville, MD). Thiol-poly(ethylene glycol)-biotin was purchased from Nanocs (New

York, NY). Ethanol, acetone, and other chemicals were purchased and used as received from VWR (Radnor, Pennsylvania).

Four different proteins were used as models to demonstrate particle bifunctionalization: BSA represents an important passivation protein, FN and immunoglobulin G antibody are important adhesive proteins, and SA is a useful cross-linking protein which binds with a high affinity to biotinylated molecules. The BSA was received fluorescently unlabeled as well as pre-labeled with FITC. The SA was also received pre-labeled with Alexa Fluor 647. FN was labeled with TRITC according to the manufacturer's instructions. Goat anti-rabbit IgG conjugated with Alexa Fluor 488 was used to later label the non-fluorescent rabbit IgG. Lyophilized proteins were typically reconstituted at 2 mg/mL, with an exception to FN which was reconstituted at 1 mg/mL, according to the manufacturer's instructions.

2.1 Monolayer Assembly

The monolayer assembly preparation involves a series of wash cycles to remove the surfactants on the particle surfaces. Particles were suspended in double distilled water followed by a 5 minute centrifuge cycle at 2500xG, 5000xG, or 10,000xG for the silica, carboxylated polystyrene, and biotin-conjugated particles respectively. The supernatant was aspirated and the particles were resuspended in double distilled water followed by another centrifuge cycle. This wash cycle was repeated three times with the particles resuspended in a final concentration that would minimize overstacking clusters when deposited onto a glass sliver. The slivers were created from glass microscope slides that were cut (Om Chigasaki TLC glass plate cutter) to fit into 2 mL microcentrifuge tubes for easier particle sedimentation and aspiration for future processing steps to increase the total yield.

The “optimal” particle concentration for monolayer preparation was determined experimentally starting with a 20 mg/mL particle concentration and spotting 1 µL and 2 µL droplets of the solution onto the glass slivers. Spots dried on the hot plate possessed particles that were more likely to form overstacking clusters and were relatively more difficult to remove via sonication compared to spots dried at room temperature.

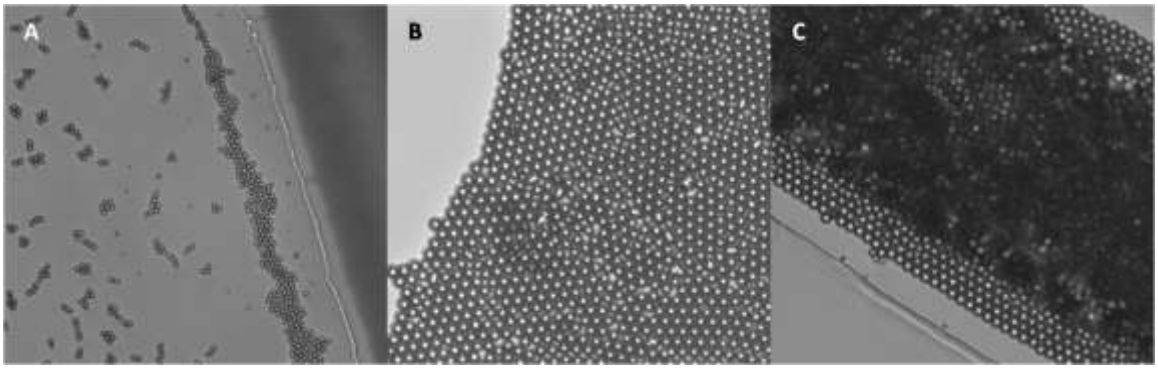


Figure 2.1. Silica particle monolayer arrangement on glass slivers. (A) Sparse monolayer of particles at the edge of droplet. (B) Optimal monolayer assembly of particles. (C) Overstacking clusters of particles at the edge of droplet.

For this reason, all spots were air-dried at room temperature and examined visually under an optical microscope with the final particle concentrations along with droplet volumes chosen to reduce the formation of overstacking clusters. These concentrations are summarized in Table 2.1. Each glass sliver had droplets that were carefully spotted and air-dried at room temperature. Any contact between the pipette tip and the glass substrate while spotting was more likely to form overstacking clusters of particles.

Table 2.1: Optimal particle concentrations for monolayer assembly.

Particle Material	Surface Reactivity	Diameter [μm]	Optimal Concentration for Monolayer [mg/mL]	Droplet Volume [μL]
Silica	None	4.74	20	2
Polystyrene	Biotin	2	0.05	1
Polystyrene	Carboxyl	2.122	0.7	1
Polystyrene	Carboxyl	4.08	0.7	1

2.2 Chemical Modification of Microparticles through Gold Deposition

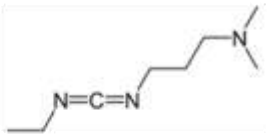
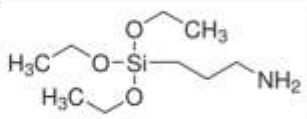
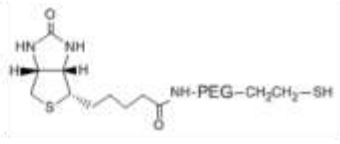
After the monolayer preparation, the particles were coated with a thin layer of gold using a metal evaporation process (CHA E-Beam Evaporator).[15] This process uses a unidirectional flux of metal deposition that permanently masks the exposed hemisphere while the respective hemisphere undergoes the shadowing effect and remains unchanged. The gold was typically deposited to a thickness of 100 nm at a rate of 2 Å/s following a 10 nm titanium adhesion layer. After the gold deposition, the glass slivers were placed into microcentrifuge tubes filled with solvent for the next processing step and were gently sonicated for 60 seconds to remove the gold-coated particles (Haier Ultrasonic Cleaner). Atomic force microscopy (AFM) imaging, scanning electron microscopy (SEM), and bright field microscopy were used to verify hemispherical gold coverage.

2.3 Protein Conjugations to the Particle Surface

The gold-coated hemisphere spatially defined a region for the selective conjugation of one type of protein onto the microparticle surface. The gold hemispherical shell was selectively modified via an amino-alkanethiol reagent[18,16,21,11,22] or thiol-PEG-

biotin.[23] The other hemisphere was modified by selectively activating the original particle surface via EDAC activation or APTES silanization. Each gold-coated particle, with the exception of those created from the biotin-conjugated polystyrene particles, was treated with a pair of selective modification reagents to create two unique chemically activated surfaces with spatially segregated proteins. The gold-coated biotin-conjugated polystyrene particles possess biotin molecules that create a functional surface through its high affinity to SA. Thus, after the gold-functionalization process, the gold-coated biotinylated particles will possess two chemically activated surfaces simultaneously. Each reagent used in this study, however, has solvent, pH, or temperature requirements that can alter protein stability towards pre-existing proteins on the particle surface prior to other functionalization processes.

Table 2.2: Cross-linkers and their respective functional group targets.

Functional Group Target	Reactivity	Cross-linker	Structure
Carboxyl	Amine	1-Ethyl-3(dimethylaminopropyl)carbodiimide (EDAC)	
Hydroxyl	Amine	3-(Aminopropyl)triethoxysilane (APTES)	
Sulfhydryl	Amine	8-Amino-1-octanethiol	
Sulfhydryl	Biotin	Thiol-PEG-biotin	

2.3.1 EDAC

EDAC is a carbodiimide cross-linker that activates exposed carboxyl groups and creates an amine-reactive ester to covalently cross-link to a protein with a primary amine group. However, the carbodiimide must be dissolved in a buffer with a pH between 4-6 in order to activate the exposed carboxylated surfaces while providing the highest yield of amide bond formations.[24] In this study, the particles were washed in a coupling buffer with a pH of 5.2 or 6.0 and typically resuspended in 100 µL of buffer for every 0.5 mg of particles. A freshly-prepared EDAC solution at a 200 mg/mL concentration was used immediately by adding 0.8µL of the EDAC solution and 10 µg of protein for every 0.5 mg of particles. The particles were gently vortexed and mixed for a one-hour incubation.

2.3.2 APTES

(3-Aminopropyl)triethoxysilane (APTES) is a silane coupling agent that activates the exposed hydroxyl groups on a surface to allow protein adsorption.[24] However, heat is typically applied in the final step of the silanization treatment to increase the binding efficiency to the inorganic surface. In this study, APTES was used to treat particles with a non-functional silica surface. The particles were washed with ethanol and air dried to ensure a clean surface prior to silanization. Then the particles were resuspended in a fresh solution of 2% APTES in ethanol for at least 30 seconds. The particles were pelleted and washed with double distilled water followed by a repeated centrifuge cycle. After the rinsing cycle, the particles are dried in an oven or on a hot plate, thus completing the silanization process. The particles were resuspended in solvent for the next processing

step followed by a quick sonication to break up any aggregated particles from the heat treatment.

2.3.3 Amino-alkanethiol

8-amino-1-octanethiol, an amino-alkanethiol, possesses a free thiol which has a high affinity to gold and therefore covalently binds to a gold surface. However, this amino-alkanethiol reagent is only soluble in potentially harsh solvents that can create protein instability and affect functionality. A 1mM solution of amino-alkanethiol in ethanol was incubated with the gold-coated particles for 2 hours to complete the gold-functionalization process. After a wash cycle, the particles were resuspended in PBS for further processing.

2.3.4 Thiol-PEG-Biotin

Thiol-PEG-biotin is a heterobifunctional PEG derivative that possesses a thiol group which has a high affinity towards gold surfaces and an exposed biotin which provides a high selectivity for SA. The PEG spacer between the reactive groups prevents unwanted non-specific adsorption to the surface during protein incubation. Thiol-PEG-biotin is also soluble in PBS which helps maintain protein stability and functionality on particles with pre-conjugated proteins. The gold-coated particles were incubated in a 1 mM thiol-PEG-biotin solution for 4 hours to complete the gold-functionalization process. After the functionalization is complete, the particles undergo a wash cycle and were resuspended in PBS for further processing.

2.4 Mixed versus Uniform Protein Surface Density

Lower densities of protein across a surface may affect the efficiency of binding or activation.[25] Therefore, the density of protein on the surface of the bifunctional Janus particles was measured and compared to particles that were not spatially segregated. Monofunctional silica and polystyrene particles, both non-gold-coated and gold coated, were functionalized with BSA. Mixtures of functionalization where equal ratios of labeled BSA and unlabeled BSA were conjugated to microparticles through EDAC cross-linking in the case of carboxylated PS microparticles or through APTES in the case of silica particles. The gold-coated particles were further processed with thiol-PEG-biotin to prevent non-specific adsorption to the gold surface.

2.5 Janus Particle Fabrication Process

The general bifunctional particle fabrication process involves several steps such as the monolayer assembly, gold deposition, particle removal, original surface modification, and the gold surface modification. The metal deposition process forms a gold hemisphere that spatially defined the selective conjugation of one type of protein onto the surface of the microparticles. This gold hemispherical shell is selectively modified via an amino-alkanethiol reagent[16,18,21,11] or thiol-PEG-biotin.[23] The other hemisphere was modified by selectively activating the original particle surface via several alternate procedures. Figure 2.2 shows a general schematic of the process flow for bifunctional particle fabrication. Gold-coated monofunctional particles were created prior to creating the bifunctional particles in order to better understand the spatial segregation of proteins on a specific surface. These gold-coated monofunctional particles were

processed with one of the previously mentioned reagents followed by a one-hour protein incubation.

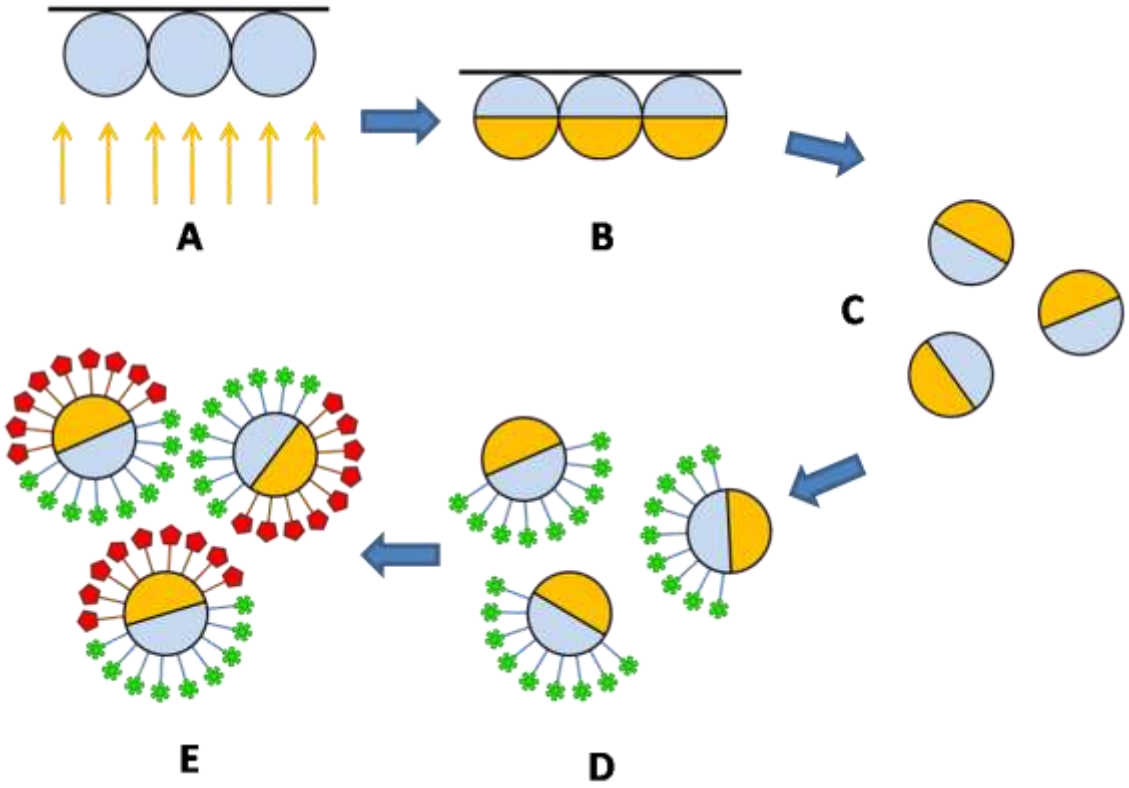


Figure 2.2. General schematic of the bifunctional Janus particle fabrication process, including (A) particles before gold deposition on the glass substrate, (B) particles after gold deposition, (C) particle removal from glass substrate, (D) original particle surface functionalization, and (E) gold functionalization.

In the fabrication of Janus particles, the first protein type was typically conjugated to the original particle surface, followed by the second protein conjugation to the gold surface. In earlier studies, silica and carboxyl polystyrene particles were functionalized with APTES and EDAC, respectively, with fluorescent BSA conjugated to the original particle surfaces via a one-hour incubation. After the original surfaces were opsonized,

the particles were processed with amino-alkanethiol followed by a one-hour incubation to conjugate FN onto the gold-coated surfaces.

Gold-coated silica particles were also processed with APTES silanization and thiol-PEG-biotin. Dissolving the thiol-PEG-biotin in a 1 mM solution in PBS allows for the particle to possess two chemically active surfaces simultaneously. As previously mentioned, the APTES selectively modified the silica surface for non-specific protein adsorption while the thiol-PEG-biotin selectively modified the gold surface and expressed a high affinity for SA. These gold-coated silica particles were processed with APTES followed by thiol-PEG biotin and vice versa to compare the differences in process flow and yields. FITC conjugated BSA or rabbit IgG (rIgG) have been conjugated onto the silica surface with fluorescently-labeled SA conjugated to the gold surface.

Thiol-PEG-biotin's solubility in PBS may allow the gold surfaces to be functionalized without affecting the functionality and stability of proteins that are pre-conjugated onto the alternate (original) surface. Thus, gold-coated carboxylated polystyrene particles were processed with EDAC and BSA followed by thiol-PEG-biotin and a SA conjugation and vice versa to compare the differences in processes and yields. The 4.08 μm diameter gold-coated polystyrene particles used in this experiment were processed with gold and stored in deionized water for at least a year prior to functionalization and protein conjugation.

2.6 Microscopy and Flow Cytometry

Confocal laser microscopy (Zeiss LSM 510 VIS Confocal Microscope) was used to visually demonstrate the spatial segregation of the two proteins onto the hemispherical surfaces of individual particles. Flow cytometry was performed using an Accuri C6 flow cytometer (BD Accuri Cytometers), and FlowJo (TreeStar, Inc.) was used to analyze the cytometry data to verify the intensity distribution of the fluorophore-conjugated proteins for a population of 100,000 microparticles. The intensity was determined over a fixed area over the fluorescent portion of each particle in the FL-1 and FL-4 channels, corresponding to the FITC/Alexa Fluor 488 and Alexa Fluor 647 emission spectra (respectively), and the forward scatter was recorded to identify debris. Three groups of controls were used consisting of gold-coated particles that were processed as previously described with thiol-PEG-biotin and APTES. The negative control microparticles were not further processed and were entirely non-fluorescent. The other two controls were processed with either FITC-BSA or Alexa Fluor 488-rIgG, interchangeably, on the original particle hemisphere, or with the Alexa Fluor 647-conjugated SA cross-linked to the gold hemisphere. These controls were used to exclude particle solution debris and identify the boundaries of fluorescence for the bifunctional Janus particles with dual fluorescence. Bifunctional particles were identified by observing significant fluorescence in both FL-1 and FL-4 channels.

CHAPTER 3

RESULTS

3.1 Particle Removal Verification

Atomic force microscopy (AFM) imaging (Asylum Research MFP-3D) was used to characterize the density of the microparticle monolayer by examining the remnant gold layer after the microparticles were removed. The density on the glass slivers with the 4.74 μm diameter silica microparticles was determined from AFM analysis to be 50,000 particles per mm^2, with a slightly lower density of particles in the central region of the monolayer. The silica gold-coated particles were further characterized with scanning electron microscopy (SEM, Hitachi S-3700 VP-SEM) to verify the hemispherical gold coverage. Figure 3.1.B and Figure 3.1.C show the hemispherical gold coverage on the silica microparticles. There are small defects at the interface between the original particle surface and the gold surface. This may be caused from a densely packed particle monolayer in which gold metal accumulates at the particle-particle interface during the gold deposition process and breaks apart during the particle removal phase and results in the defects.

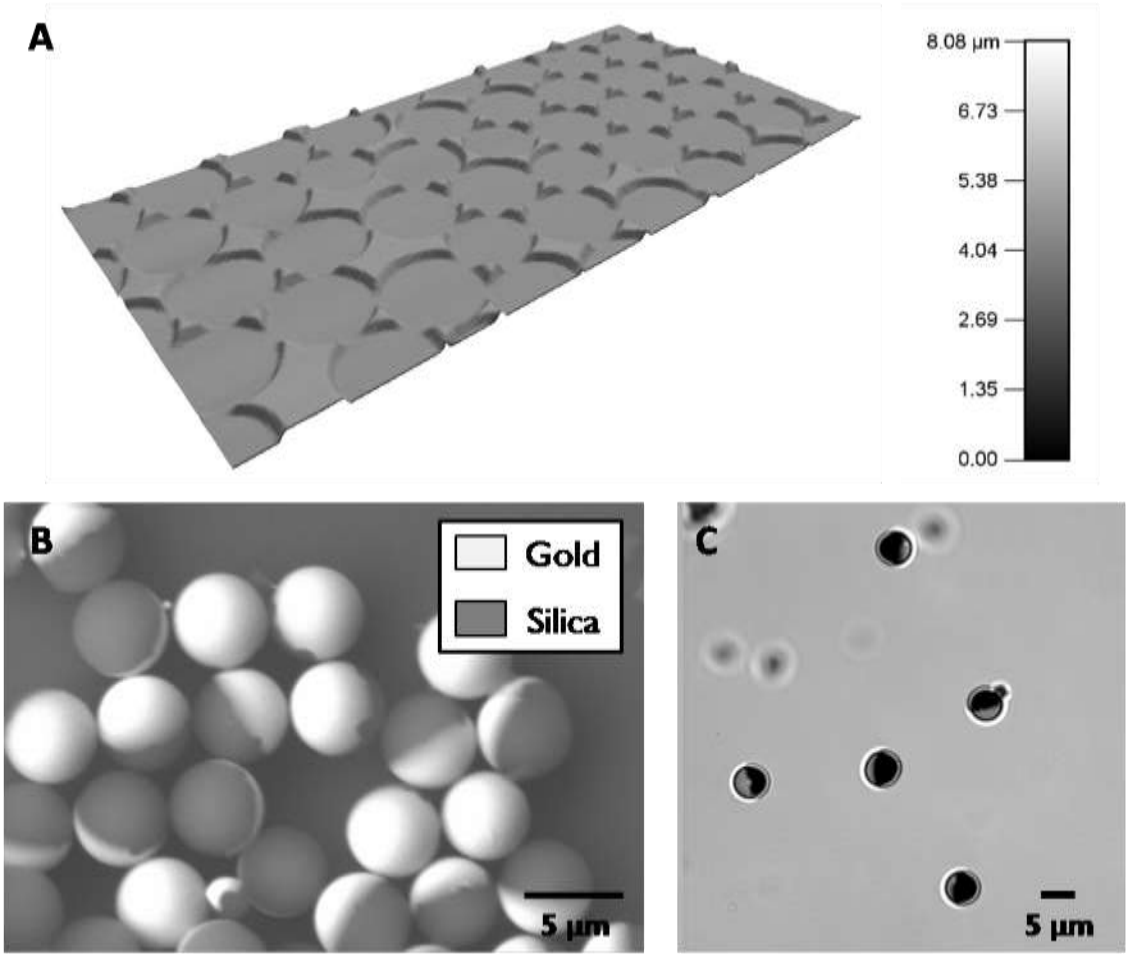


Figure 3.1. Gold-coated particle removal verification and characterization. (A) AFM surface topography of glass substrate after particle removal, (B) SEM of 4.7 µm gold-coated silica particles after removal by sonication of the glass substrate, and (C) bright field microscopy of the particles.

3.2 Confocal Microscopy

Monofunctional particles with FITC conjugated BSA spatially segregated onto the silica or gold hemisphere via APTES or amino-alkanethiol respectively were imaged using the confocal laser microscope as shown in Figure 3.2. A variety of Janus particles were created with distinct protein types spatially segregated onto two hemispheres. All particles were processed and imaged with confocal laser scanning microscopy and bright field imaging. The observed fluorescent regions correspond to the microparticles' derivatized hemispheres which indicates the successfully bifunctionalized, segregated surfaces (Figure 3.6).

Figure 3.3 shows particles with spatially segregated proteins. However, there was inconsistent protein coverage on the gold hemispherical surfaces among the different particle material types used in this study. For this reason, thiol-PEG-biotin, another gold functionlization reagent, was chosen to replace amino-alkanethiol in the gold functionalization step of the fabrication process. Gold-coated polystyrene particles (2 μm and 4.08μm diameters) processed with EDAC and thiol-PEG-biotin demonstrate the sucessful spatial segregation of proteins . Yet, the fluorescent signal from the fluorophore-conjugated proteins on the carboxylated hemisphere is relatively weaker compared to the fluorescent signal previously seen on the monofunctional gold-cated silica particles processed with APTES silanization (Figure 3.2.B). Gold-coated silica particles processed with APTES silanization and thiol-PEG-biotin functionalization demonstrate the spatial segregation of proteins with relatively strong fluorescent signals in comparison to Figure 3.4. Figure 3.6 demonstrates the material selectivity in the

APTES silanization and thiol-PEG-biotin functionalization processes, with clear spatial definition between the proteins.

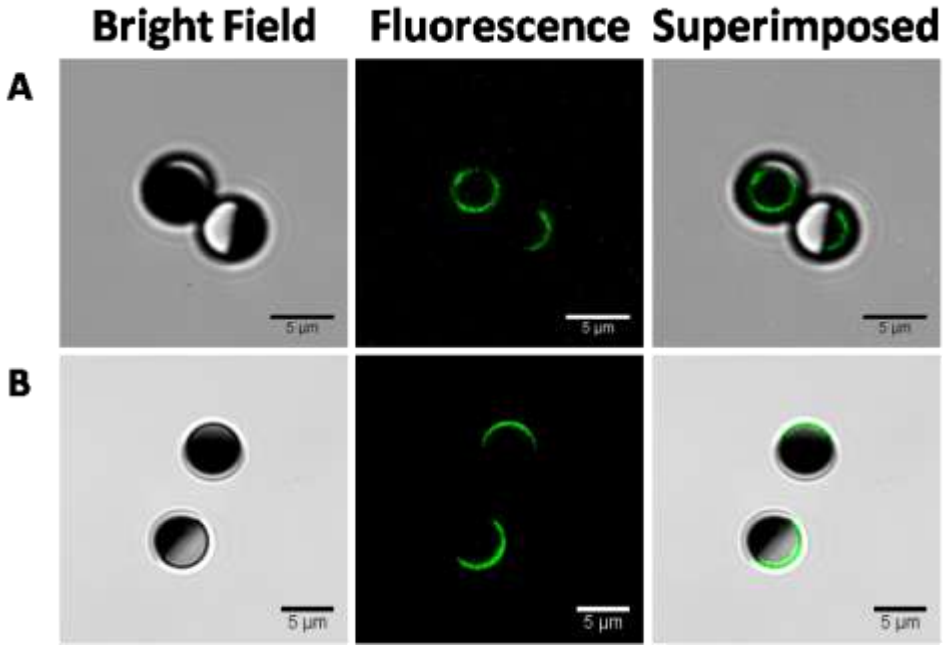


Figure 3.2. Monofunctionalized silica particles processed with (A) amino-alkane thiol and (B) APTES silanization and conjugated with Alexa Fluor 488-conjugated BSA.

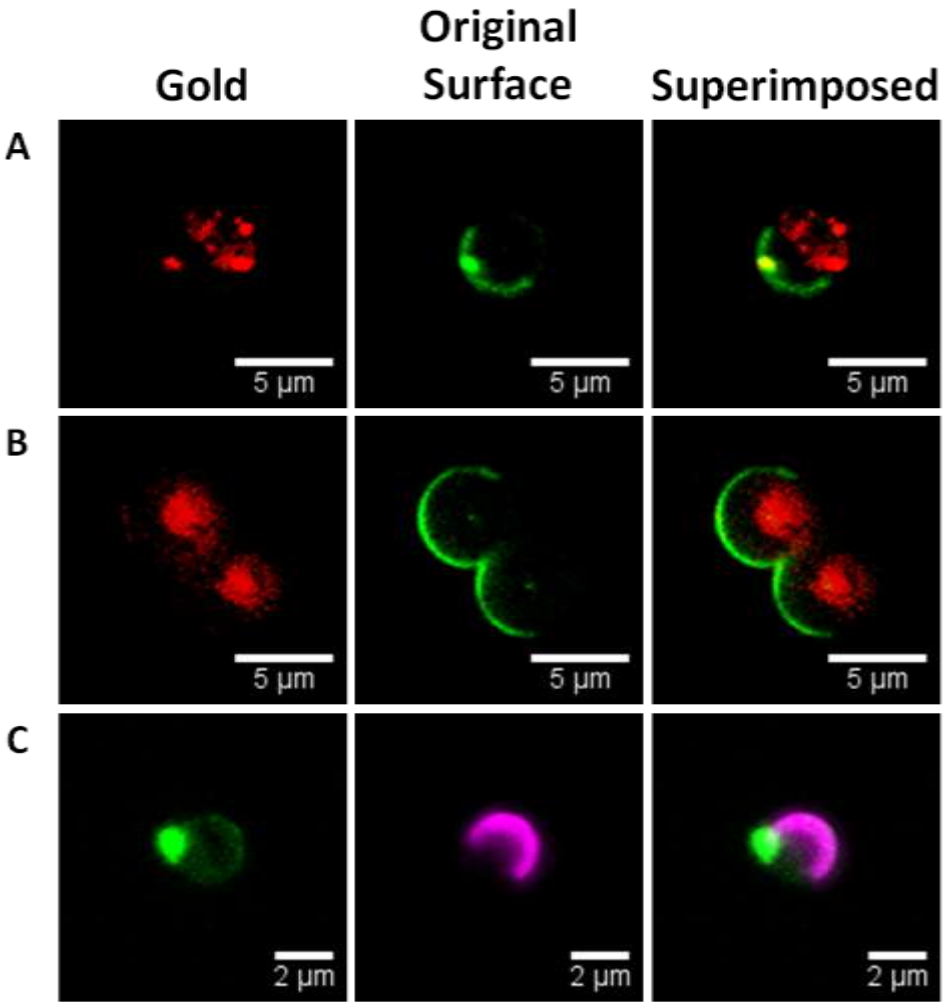


Figure 3.3. Confocal images of bifunctional Janus particles created from various materials with amino-alkanethiol processing. (A) Carboxylated polystyrene particle (diameter = 4.08 µm) processed with EDAC and amino-alkanethiol followed with FITC conjugated BSA and TRITC conjugated FN cross-linking onto the polystyrene and gold surfaces respectively. (B) Silica particles (diameter = 4.74 µm) processed with APTES and amino-alkanethiol followed with FITC conjugated BSA and TRITC conjugated FN cross-linking onto the silica and gold surfaces respectively, and (C) biotin-conjugated polystyrene particle (diameter = 2 µm) processed with amino-alkanethiol followed with FITC conjugated BSA and SA cross-linking onto the gold and biotinylated surfaces respectively.

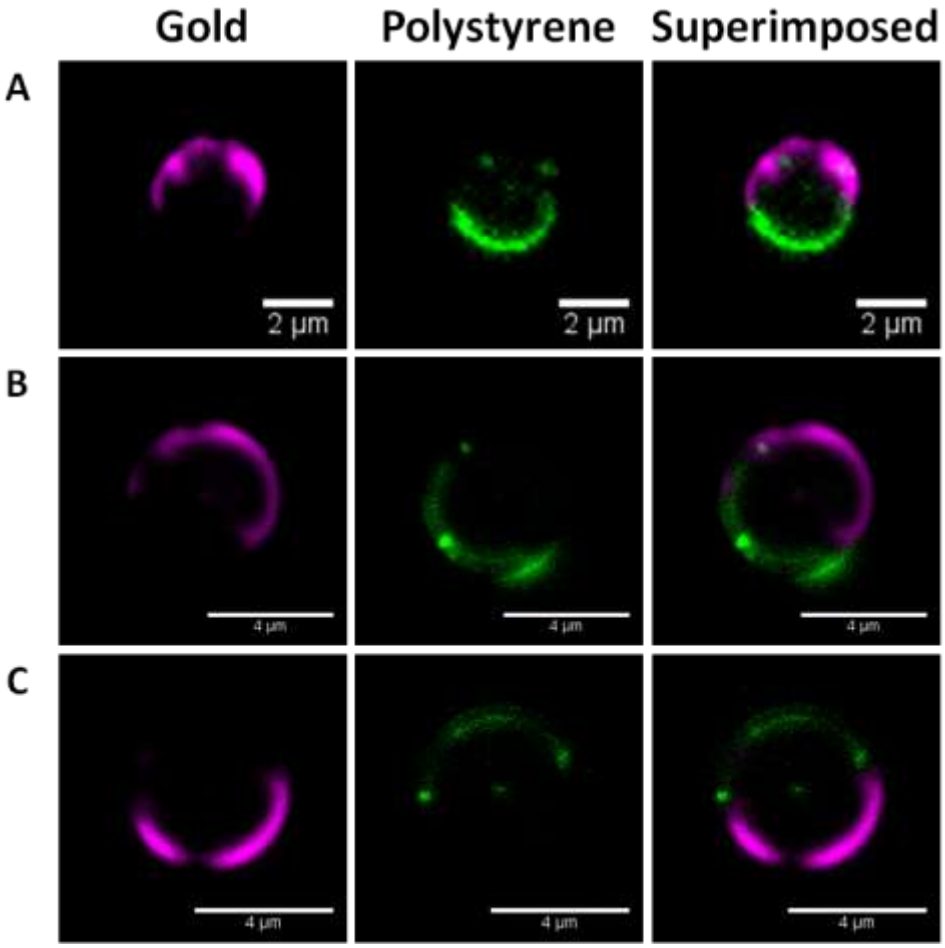


Figure 3.4. Confocal images of bifunctional Janus particles created from carboxylated polystyrene particles with thiol-PEG-biotin processing. (A) Particles (diameter = 2 µm) processed with EDAC and FITC conjugated BSA followed with thiol-PEG-biotin and SA cross-linking onto the polystyrene and gold surfaces respectively. (B) Particles (diameter = 4.08 µm) processed with EDAC and FITC conjugated BSA followed with thiol-PEG-biotin and SA cross-linking onto the polystyrene and gold surfaces respectively, and (C) Particles (diameter = 4.08 µm) processed with thiol-PEG-biotin followed by EDAC and FITC conjugated BSA and SA cross-linking onto the polystyrene and gold surfaces respectively.

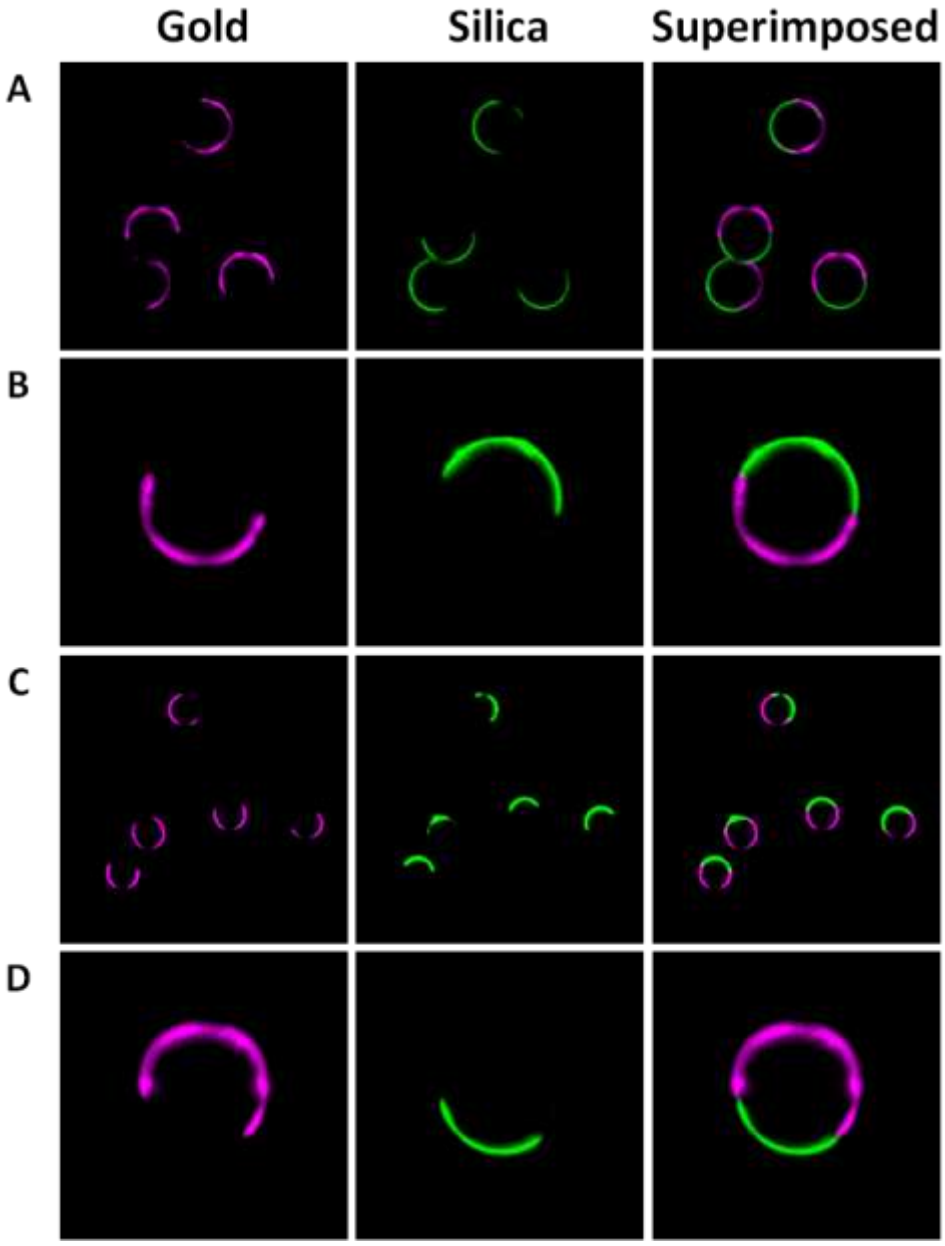


Figure 3.5. Confocal images of 4.74 μm silica particles processed with thiol-PEG-biotin and APTES to create bifunctional Janus particles. (A) Overall and (B) magnified view of particles with FITC conjugated BSA and Alexa Fluor 647 conjugated SA on silica and gold surfaces respectively. (C) Overview and (D) magnified view of particles with Alexa Fluor 488 conjugated goat anti-rabbit IgG and Alexa Fluor 647 conjugated SA on silica and gold surfaces respectively.

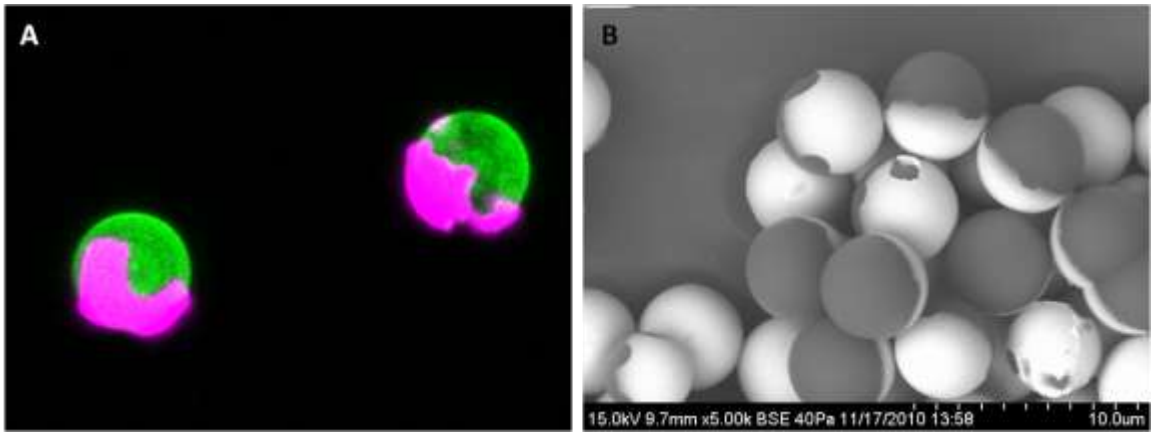


Figure 3.6. Comparison of protein spatial segregation onto different surfaces particles to gold-coated silica particles (diameter = 4.74 µm) with similar hemispherical gold coverage. (A) 3D Confocal image of bifunctional Janus particles created from gold-coated silica particles processed with APTES/thiol-PEG-biotin and conjugated with BSA and SA spatially segregated. (B) SEM image of gold-coated silica particles with show similar gold coverage as (A).

Figure 3.7. Rotating silica microparticle (diameter = 4.74 µm) processed with thiol-PEG-biotin/APTES followed by BSA/SA cross-linking onto the silica and gold surfaces respectively. (tang_jennifer_l_201205_mast_rotating_particle.avi, 145,301K)

The protein surface density of the bifunctional Janus particles was measured and compared to the particles which did not possess spatially segregated proteins. Mean fluorescence intensity (MFI) was measured using Zen Lite 2011 as a surrogate for protein density measurements on the surface of the gold-coated particles with a single protein type and the microparticles functionalized with a mixture of labeled and unlabeled BSA. A two-sample T-test (Minitab) demonstrates significance between the fully-coated microparticles opsonized with labeled BSA and the fully-coated microparticles opsonized with an equal mixture of labeled and unlabeled BSA. There was no statistical significance in the MFI among the fluorescent regions of the fully-coated microparticles and the gold-

coated particles that were conjugated with labeled BSA. This result supports the visual data indicating that the density of proteins is equivalent on the functionalized hemispheres of the Janus particles and the fully coated particles, but not in the diluted mixture. Spatial segregation of protein functionalization therefore is important to maintain a high density of ligands in the creation of bifunctional particles. This should improve the biological activity of the bifunctional Janus particles due to multivalent interactions that will increase interaction efficiency or may be required for some biological effects.

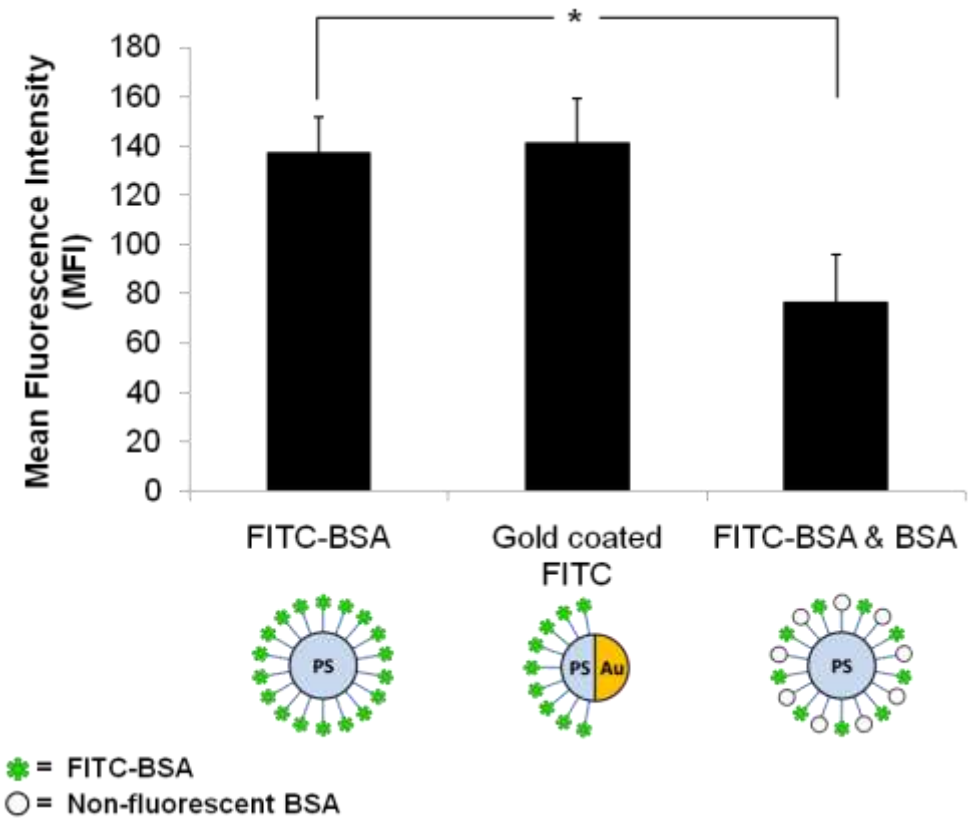


Figure 3.8. Comparison of the MFI of carboxylated polystyrene particles coated with FITC conjugated BSA or an equal mixture of FITC conjugated BSA and unlabeled BSA. Measurements are shown for single protein coating, mixed protein coating, and single protein Janus particles as depicted by the schematics below each box plot. The mixed protein coating consists of FITC conjugated BSA and unlabeled BSA.

3.3 Flow Cytometry

Flow cytometry was used to quantify the yield of bifunctional particle production from each processing method. Two yields were examined. The first is the process yield that measures the percentage of particles successfully functionalized with two types of spatially segregated proteins compared to the total number of particles present after the processing has been completed, as determined by flow cytometric analysis. Experiments were also performed to test whether there were significant differences in the process yields of the silica particles functionalized with APTES before or after thiol-PEG-biotin gold-functionalization. Analysis of the flow cytometric results shows that the gold-coated silica particles processed with thiol-PEG-biotin followed by APTES silanization provided a slightly higher process yield compared to particles processed with thiol-PEG-biotin after APTES silanization. For this reason, silica particles were processed with thiol-PEG-biotin followed by APTES silanization and rIgG/SA conjugations, which resulted in a 90.5% process yield. Due to the small sample size of carboxylated polystyrene particles processed with EDAC and thiol-PEG-biotin, the process yield could not be determined. The overall different process yields are summarized in Table 3.1.

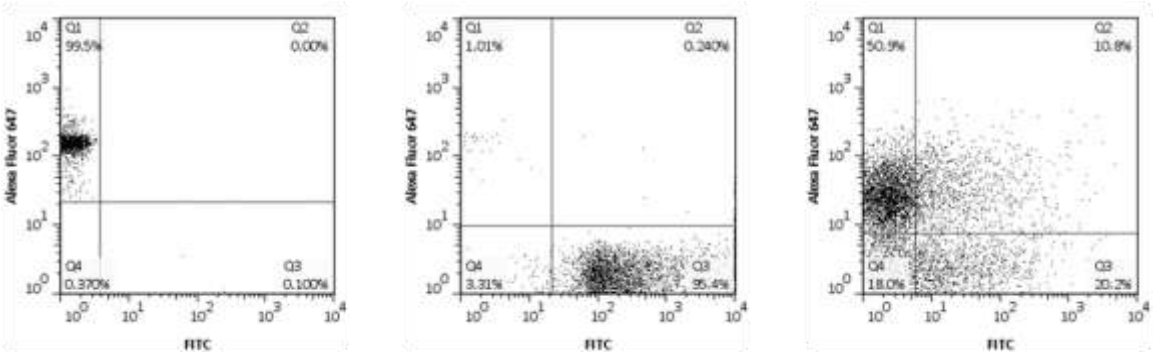


Figure 3.9. Fluorescence analysis of bifunctional Janus particles using flow cytometry for biotinylated particles processed with amino-alkanethiol with BSA and SA conjugated onto the gold and biotinylated surfaces respectively.

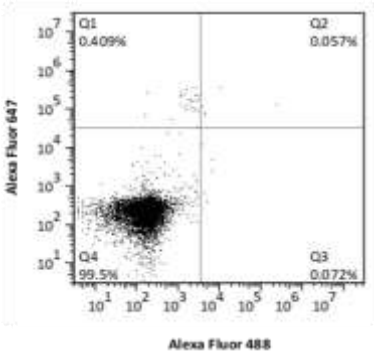


Figure 3.10. Fluorescence analysis of (unlabeled) negative control for Janus particles using flow cytometry for silica particles processed with thiol-PEG-biotin followed by APTES silanization.

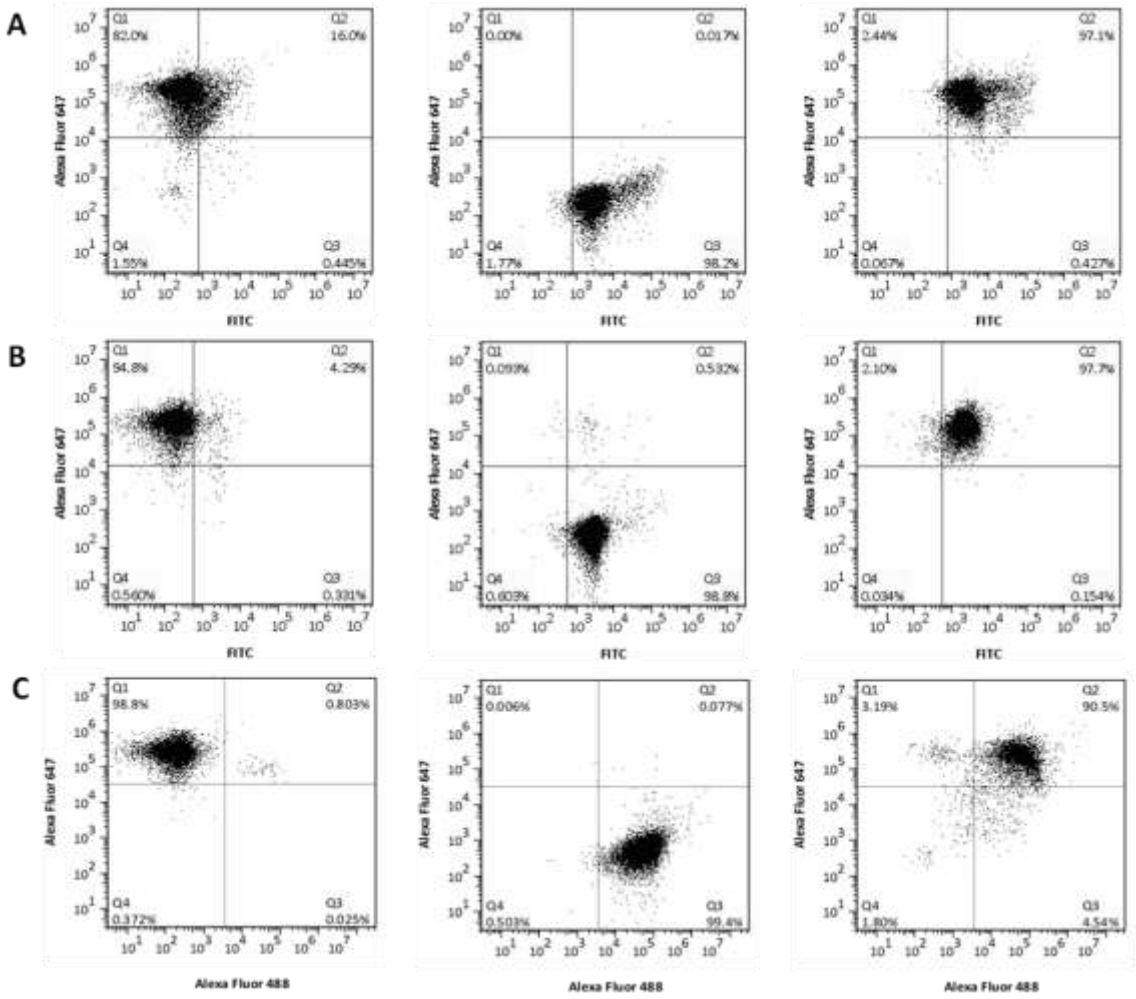


Figure 3.10. Fluorescence analysis of bifunctional Janus particles using flow cytometery for silica particles processed with (A) APTES silanization followed by thiol-PEG-biotin and BSA/SA conjugation. Silica particles were also processed with thiol-PEG-biotin functionalization followed by APTES silanization and (B) BSA/SA and (C) rIgG/SA conjugations.

The second type is the total yield and is defined as the number of bifunctional particles created compared to the initial number of particles that were used prior to processing. This number is by definition lower since some particles are lost in the gold deposition and washing steps. The total yield is approximately 25% for all processing methods. The total yield for the silica and carboxylated polystyrene particles processed with amino-alkanethiol and FN/BSA showed a significantly lower yield with not enough particles to analyze with the flow cytometer. The yield of the FN/BSA conjugated particles can be optimized by decreasing the agitation during the FN conjugation step and pre-rinsing the microcentrifuge tubes with a 0.1% Tween solution to prevent particle and protein adsorption.

Table 3.1 Summarized results for different procedures of bifunctional Janus fabrication process. We conjugated BSA/SA and rIgG/SA to the silica/gold microparticles via APTES/Thiol-PEG-biotin cross-linking chemistry, which provided a range in process yield.

Particle Surface	**Cross-linking Chemistry**	**Process Yield**
Carboxylated Polystyrene/Gold	EDAC/Amino-alkanethiol	< 5%
Biotin-conjugated Polystyrene/Gold	None/Amino-alkanethiol	< 5%
Silica/Gold	APTES/Amino-alkanethiol	11%
Carboxylated Polystyrene/Gold	EDAC/Thiol-PEG-biotin	< 10%
Silica/Gold	APTES/Thiol-PEG-biotin	90.5-98%

CHAPTER 4

DISCUSSION

As seen from the process yield results, silica particles processed with the thiol-PEG-biotin/APTES functionalization processes provide the highest yield of bifunctional bifunctional Janus particles. This may be in part due to the PEG spacer inhibiting non-specific adsorption to the gold surface while biotinylation exhibits a high selectivity for SA. For all successful functionalizations regardless of yield, the microparticles have two selective surfaces to which proteins can form a stable bond.

Utilizing thiol-PEG-biotin allows both the silica hemispherical surfaces and the gold coatings to both be chemically active prior to protein immobilization. Protein instability in potentially harsh solvents or conditions is therefore avoided during all subsequent protein conjugation steps. This helps to maintain the native conformation of proteins as they are conjugated to the particle surfaces. Also, this procedure helps to increase the total yield due to fewer particle processing steps.

There are noticeable differences in the fluorescent signals among the particles processed with thiol-PEG-biotin. The carboxylated polystyrene particles, in comparison to the silica particles, possess a weaker signal that photobleached rapidly during the image scanning. This signal may be improved with longer incubation times to allow for better cross-linking between the proteins and the carboxylated surface. Also, the larger polystyrene particles in Figure 3.5 were stored in deionized water for over a year, where

the carboxyl surface of the original particle material may be affected by contaminants within the water over time. In all cases of carboxylated polystyrene particles processed with thiol-PEG-biotin, SA was observed to be spatially segregated as indicated by the Alexa Fluor 647 fluorophores on the biotinylated surface.

A higher total yield of spatially segregated proteins was achieved using the silica particles compared with the polystyrene particles. The lower total yield for polystyrene particles may be a result of increased nonspecific binding caused by the inherent surface charge of the material as well as the hydrophobic nature of polystyrene. Carboxylated polystyrene particle aggregation was observed during the EDAC conjugation, especially for particles with low carboxyl densities on the surface. The microcentrifuge tubes can be rinsed with 0.1% Tween, a surfactant solution, prior to EDAC activation to allow for easier particle sedimentation. Similarly, a 0.0005% Tween solution in the coupling buffer can be used to reduce particle aggregation during the EDAC activation. Despite polystyrene's hydrophobic nature, the biotin-conjugated polystyrene particles have a relatively higher process yield of 11%, most likely due to the high affinity binding to SA and lower nonspecific adsorption.

The MFI distribution used to compare the proteins across a single or spatially segregated surface demonstrates that particles with spatially segregated proteins do not possess a significant difference with non-gold-coated particles coated with a single ligand type. However, the MFI measurements from the fluorescent portions of the microparticles showed a statistical significance among the non-gold-coated particles labeled with either fluorescent BSA or with an equal mixture of fluorescent and non-fluorescent BSA. The significant difference in fluorescence was expected due to the

different populations of mixed protein solutions with fluorescent and non-fluorescent protein.[26] Other flow cytometry data may have showed two populations which may be caused from sonication for particle removal in which clusters of microparticles did not break apart or the gold remnants on the glass slide were removed from the glass substrate as well (Figure 3.1.A). These larger gold fragments may not have been filtered and were later functionalized with thiol-PEG-biotin and cross-linked with the fluorophore conjugated SA.

CHAPTER 5

CONCLUSION

Bifunctional Janus particles have been created with biologically relevant, native conformation proteins covalently linked to a biologically-unreactive and safe substrate.[18,21] Depending on the protein types conjugated onto the surfaces, these particles can be integrated into many biomedical applications, including multifunctional drug targeting, cell propulsion, bioimaging, and adhesive cross-linking of cells. For example, bifunctional Janus particles can impart separate physically linked recognition and activation capabilities that can be used in drug targeting. In this case, a recognition protein targets and attaches itself to a specific cell, large protein complex, or infectious agent, while the activation moiety stimulates an immune response to start killing and breaking down the unwanted target. The high density of proteins on the functionalized surface could also increase selectivity that could then reduce the number or magnitude of side effects from pharmaceutical drugs. With these Janus particles, drug delivery systems may improve in range, specificity, and capabilities for therapeutic relief.

These studies were conducted as a "proof of principle" for the creation of bifunctional Janus particles. By attaching fluorophore-conjugated proteins onto two chemically-distinct hemispheres of a microparticle, we showed that two distinct proteins can be deposited onto a microparticle, creating bifunctional capabilities. Creating bifunctional microparticles with a high density of proteins can be important in the development of more advanced therapeutic particles by incorporating multiple targeting

and stimulation capabilities in one entity or adding a therapeutic capability imparted by one or more protein types to each hemisphere. Spatially segregated bifunctionality, in comparison to mixed distributions on a surface, leads to a higher density of ligands that may increase adhesive capabilities through multivalent binding[26] or improve signaling capabilities through cross-linking of receptors or other sterically-sensitive processes. Bifunctionality can therefore improve the effectiveness of microparticle technologies for applications where multifunctionality and high avidity are necessary.

For example, these bifunctional capabilities can be integrated into phagocytosis studies to better understand the phagocytotic nature of cells. One method is to observe complement activation via vary densities of F_c receptors on an exposed surface. This uses the zipper model of F_c receptor mediated phagocytotic cells. This model states that these cells form a cup-like depression on the surface of the cell after initial binding, with each subsequent binding between the ligand/receptor "zippering" the cup over the particle until it is engulfed. After recognition, this is the essential first step of phagocytosis.[27] However, in this model, the targets are fully and uniformly coated with ligands, and whether particles displaying spatially segregated ligands onto only one hemisphere would stimulate phagocytosis remains unknown. Studies observing phagocytotic response to a half-coated particle can be conducted to determine whether the particles will be completely engulfed within the cell or whether the PEG spacer length affects this response.

All of the bifunctional particles fabricated in this study were created as a "proof of principle" concept to demonstrate the spatial segregation of proteins and are in the microscale range of 2 μm to 4.74 μm diameters. However, a smaller size is important for

many biological processes, such as enhanced permeability retention (EPR). EPR uses the tumor vascularization process that may allow for the particles to leak through poorly lined epithelial cells in the blood vessels.[28]

The bifunctional particles in this study utilized thiol chemistry via amino-alkanethiol and thiol-PEG-biotin to functionalize the gold surfaces. However, it is important to demonstrate the universal cross-linking of proteins onto spatially segregated surfaces. This study may be conducted through the utilization of histidine-tagged proteins which can control proteins orientation while possessing a high specificity to other metals and may be used with nickel or copper surfaces.[29] This fabrication technique with nickel, copper, and histidine-tagged proteins may allow for improved targeting efficiency with via the controlled orientation of proteins that are immobilized on the surfaces.

The general fabrication process can be modified to deposit a thin gold layer at an angle, which will provide control over the spatial coverage for each conjugation moiety. The variance in coverage may also allow for other opportunities to chemically activate certain surfaces and create multifunctional particles. Various suspension methods, such as shaking or using a stir bar, during incubation can affect the funtionalization or protein coverage on the surface, which would then affect the process and total yield. Longer incubation times may improve the cross-linking of protein to a surface. Studies to compare the effect of various PEG spacer lengths may be needed to optimize the protein coverage on the surface. Thus, all of these methods may need to be optimized to increase the process and total yield of bifunctional particles with spatially segregated proteins in future studies.

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