Bioinspired Polymeric High-Aspect-Ratio Particles with Asymmetric Janus Functionalities

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Polymeric particles with intricate morphologies and properties have been developed based on bioinspired designs for applications in regenerative medicine, tissue engineering, and drug delivery. However, the fabrication of particles with asymmetric functionalities remains a challenge. Janus polymeric particles are an emerging class of materials with asymmetric functionalities; however, they are predominantly spherical in morphology, made from nonbiocompatible materials, and made using specialized fabrication techniques. Herein nonspherical Janus particles inspired by high-aspect-ratio filamentous bacteriophage are fabricated using polycaprolactone polymers and standard methods. Janus high-aspect-ratio particles (J-HARPs) are fabricated with a nanotemplating technique to create branching morphologies selectively at one edge of the particle. J-HARPs are fabricated with maleimide handles and modified with biomolecules such as proteins and biotin. Regioselective modification is observed at the tips of J-HARPs, likely due to the increased surface area of the branching regions. Biotinylated J-HARPs demonstrate cancer cell biotin receptor targeting, as well as directional crosslinking with spherical particles via biotin–streptavidin interactions. Finally, maleimide J-HARPs are functionalized during templating to contain amines exclusively at the branching regions and are dual-labeled orthogonally, demonstrating spatially separated bioconjugation. Thus, J-HARPs represent a new class of bioinspired Janus materials with excellent regional control over biofunctionalization.

1. Introduction

Bioinspired materials have seen increasing use for applications in nanotechnology and biomedicine. Intricate materials have been reported using systems inspired by naturally occurring bioadhesives, light-harvesting systems, and self-assembling biomaterials, among others.[1–6] Bioinspired polymeric materials and particles have been engineered with diverse morphologies and physicochemical properties for applications such as tissue engineering and drug delivery.[7,8] However, the design of polymeric particles with asymmetric distributions of functionalities remains a challenge. Many examples of materials with asymmetric regions of functionality are found throughout natural systems.[9] Proteins have regiospecific functionalities and biochemical properties, which lead to controlled assembly dynamics, enzymatic active sites, and specific binding domains of antibodies.[10,11] Viruses such as filamentous bacteriophage contain multivalent displays of peptides on one end of the virus particle to enable directional binding to targets.[12] Such natural systems have been engineered by researchers to create protein-based materials with regiospecific bioconjugate chemistries, or to create biotechnologies such as enzyme-linked immunosorbent assays and phage display.[9–13] However, asymmetric biofunctionalities remain difficult to achieve using exclusively synthetic materials.

Janus particles are an emerging class of synthetic materials with asymmetric properties that have shown promise for applications in drug delivery, diagnostic medicine, and tissue engineering.[14,15] Janus materials have attracted attention for their ability to display multiple biofunctional handles with regional control to enable improved material biointerfaces[16,17] as cells can respond to biological cues in gradient-dependent manners.[17,18] In the field of drug delivery, Janus particles have shown promise in dual drug delivery, enabling the codelivery of multiple bioactive compounds with independent rates of release.[19–21] "Theranostic" Janus materials have attracted attention for their abilities to better control multiple cargo conjugations onto a single particle carrier.[15,22] Also within the field of drug delivery, Janus particles have enabled the development of micro- and nanomotors, with gas-producing catalysts at one end of a particle to enable directional active motion and the penetration of particles through biological barriers.[23] These Janus particles can be made from either inorganic or polymeric materials and fabricated through a variety of methods, including modification of particles at surface interfaces, directed material assemblies, microfluidics, electrojetting, and biphasic Pickering emulsions.[14,15,24,25] Although some Janus particles have been developed using hybrid platforms of biodegradable synthetic
and biologically derived materials, the majority of Janus particles are spherical in morphology and composed of inorganic nonbiodegradable materials or specially synthesized polymers. We therefore set out to develop, using standard fabrication techniques and commercially available biodegradable polymers, a new class of nonspherical Janus particles inspired by the regional functionalities of high-aspect-ratio filamentous bacteriophage.

To create these bacteriophage-inspired Janus particles, we utilized polycaprolactone (PCL) high-aspect-ratio particles (HARPs), an emerging class of materials which we recently reported on.[27] By adapting the HARP nanotemplating fabrication process,[27] asymmetric Janus HARPs (J-HARPs) were fabricated with high-surface-area branching morphologies at one edge of the particles. J-HARPs bearing maleimide conjugation handles were fabricated and functionalized with biomolecules such as proteins and biotin, with predominant modification observed at the branched region of the J-HARPs due to its increased surface area. We further demonstrated the versatility of J-HARPs by fabricating particles with separate regions of orthogonal reactivities and regioselectively labeled J-HARPs with two different fluorescent cargos. J-HARPs therefore represent a novel class of bioinspired materials made from readily available and biodegradable components and could see use in diverse biomedical applications.

2. Results and Discussion

2.1. Fabrication and Regioselective Labeling of J-HARPs

J-HARPs were fabricated from biodegradable PCL polymers using a nanotemplating technique. We previously reported on the use of nanotemplating to fabricate symmetric PCL HARPs of 10–20 μm length and 200 nm diameter.[27,29] PCL was spun cast into films and heated in contact with 20 nm porous anodic aluminum oxide (AAO) Anodisc templates, causing melted PCL to pass through the 20 nm branched porous region of the Anodiscs. We therefore hypothesized that if a limited templating occurs, PCL polymers would be retained partially within the 20 nm branching porous region of the AAO template (Figure 1a). Upon solidification, AAO etching, and HARP purification, particles would then bear those branching morphologies at one end, resembling the morphologies of high-aspect-ratio filamentous bacteriophage. Fabrication conditions were screened to facilitate limited templating and J-HARP formation, with optimized conditions observed using 10 kDa PCL templated at 60 °C for 30 min prior to removal of the Anodiscs from heat and subsequent cooling and etching. Helium ion microscopy (HIM) revealed asymmetric J-HARPs with a branched morphology observed exclusively at one edge of the particles (Figure 1b).

To engineer J-HARPs for biomolecule functionalization, PCL₂₅–maleimide[27] was incorporated at 20 wt% into the J-HARP backbone during fabrication. Fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA) was used to test material bioconjugation efficiencies (Figure 2a) as BSA contains a reactive thiol to facilitate maleimide–thiol addition.[31] Interestingly, predominant modification was observed at one edge of the J-HARPs, corresponding to the end with branching morphologies (Figure 2b,c). This is likely due to the higher surface area on the branching region, which can increase the exposure of maleimide groups and subsequent bioconjugation efficiencies.[32,33] The regiospecific branching at one edge of the J-HARP therefore enabled asymmetric modification of the particle in a gradient manner, with highest degrees of FITC-BSA observed at the branched region. Modification was quantified through analysis of fluorescence intensities along the length of the J-HARPs, and a steep gradient of modification was observed starting at ≈1 μm from the J-HARP edge (Figure 2d), corresponding to the point at which branching morphologies begin to be observed on the particles. A set of symmetric HARPs with maleimide handles was fabricated by templating PCL from the 200 nm side of the AAO template and reacted with FITC-BSA (Figure S3, Supporting Information). As expected, symmetric modification with no detectable fluorescence gradient was observed for symmetric HARPs due to the lack of high-surface-area regions on the particles. In addition, minimal nonspecific adsorption of FITC-BSA to J-HARPs lacking maleimide handles was observed (Figure S4, Supporting Information).

![Figure 1](https://www.advancedsciencenews.com/si-delivery/2000057/si-s1.jpg)

**Figure 1.** Fabrication and characterization of J-HARPs. a) J-HARPs were fabricated using a nanotemplating technique. Partial templating at the 20 nm side of a porous AAO template leads to asymmetric HARP formation, with branching morphologies at one end of the particle. b) HIM images of J-HARPs, confirming asymmetric branching. Larger HIM images are available in the Supporting Information.
confirming that the maleimide–thiol reaction led to the observed asymmetric fluorescence. Thus, J-HARPs have not only asymmetric morphologies, but also regiospecific reactive handles for bioconjugation in a gradient manner.

2.2. Biotinylated J-HARPs for Cancer Targeting and Directed Colloidal Assemblies

To enable the binding of filamentous phage-inspired J-HARPs were modified with heterobifunctional poly(ethylene glycol) (PEG) polymers of thiol-PEG₃k-biotin to enable the multivalent display of biotin at the branched edge of J-HARPs. J-HARPs were also fabricated to include Nile red fluorophores embedded within the PCL core for fluorescence tracking of particles. Biotinylation was confirmed through the incubation of J-HARPs with Cy5-modified streptavidin (STV). STV binding to J-HARPs was observed predominantly at the edge of the particles (Figure 3a–c). The high regioselective gradient of STV binding is likely due to the combination of initial thiol–biotin gradient formation, similar to that observed with FITC-BSA conjugation, followed by an additional binding step of biotin with STV.

Biotin receptors are overexpressed on a variety of cancer cell types, and thus biotin is a promising biomolecule to facilitate cancer targeting for applications in diagnostic medicine and drug delivery.[34,35] To determine whether biotin J-HARPs can act as cancer cell–targeting particles, A549 lung cancer cells that overexpress biotin receptors[35,36] were incubated with either non-functionalized fluorescent J-HARPs or biotinylated fluorescent J-HARPs. We observed strong interactions between biotin J-HARPs and A549 cancer cells (Figure 3d), whereas moderate interactions were observed for unmodified J-HARP controls (Figure S5, Supporting Information). Binding efficiencies were quantified, and increased cancer cell targeting was observed for biotin J-HARPs compared to unmodified J-HARPs (Figure 3e, \( p = 0.0027 \)). To confirm that binding was mediated through biotin receptor targeting, HEK-293 cells, which have lower levels

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**Figure 2.** Asymmetric functionalization of J-HARPs. a) J-HARPs were fabricated containing 20 wt% PCL–maleimide. Reaction of maleimide J-HARPs with FITC-BSA yielded BSA-conjugated J-HARPs, with maximal conjugation observed at the branched region of the particles. Asymmetric modification was confirmed via fluorescence microscopy images of FITC-BSA J-HARPs at b) 20× and c) 100× magnification. Larger fluorescence microscopy images are available in the Supporting Information. d) Quantification of J-HARP modification via analysis of fluorescence intensity across the length of J-HARPs (n = 20).

**Figure 3.** Formation of biotin J-HARPs for cancer cell targeting. J-HARPs with maleimide handles were fabricated with Nile red dye encapsulated in the PCL core and were modified with thiol–PEG₃k–biotin. a) Incubation of biotin J-HARPs with Cy5 Streptavidin (STV, green) revealed asymmetric STV binding to J-HARPs in a gradient manner. b) Nile red fluorescence in pink and c) overlay of the fluorophores reveal homogenous Nile red distribution with asymmetric biotinylation of J-HARPs. d) Confocal fluorescence microscopy z-axis projection of biotin J-HARPs incubated with lung cancer A549 cells, which overexpress biotin receptors. Strong interactions and binding events were observed between A549 cells and biotin J-HARPs. A549 cells were stained with CellMask Deep Red Plasma membrane stain (green), and J-HARPs were fabricated to contain Nile red fluorophores (pink). e) Binding efficiencies of J-HARPs for A549 cells (biotin receptor positive) and HEK-293 cells (biotin receptor negative) were quantified, and increased cancer cell targeting was observed for biotin J-HARPs to A549 cells when compared to unmodified J-HARPs (\( **p = 0.0027 \)) or when incubated with HEK-293 cells (\( *p = 0.037 \)).
of biotin receptor expression,\cite{36} were incubated with both unmodified and biotinylated J-HARPs. Reduced binding was observed for biotin J-HARPs to HEK-293 cells compared to A549 cells (p = 0.037), thus indicating that the observed increase in cell binding by biotin J-HARPs is due to receptor interactions.

In addition to facilitating targeted binding to cancer cells, biotinylated J-HARPs were also evaluated for their ability to form directional crosslinks with spherical particles through biotin-streptavidin interactions. Fluorescent polymeric microparticles were fabricated from poly(lactic-co-glycolic acid) (PLGA) and biotin–PEG–PLGA block copolymers. Biotin J-HARPs were incubated with STV to form STV J-HARPs, washed to remove excess STV, and then incubated with spherical biotin microparticles (Figure 4a). Interactions between STV-functionalized J-HARPs and biotin microparticles were observed via fluorescence microscopy (Figure 4b), with directional binding predominantly occurring at the tip of the J-HARPs. J-HARPs therefore show promise in enabling directional crosslinking and will be further engineered to facilitate controllable assembly dynamics for mixed-particle assemblies, which have demonstrated potential for applications in tissue engineering and regenerative medicine.\cite{29,37}

2.3. Orthogonal Dual Modification of Bifunctional J-HARPs

One of the features of biological materials most difficult to create using synthetic polymers is heterogeneous regions of chemical functionality. To create Janus materials with regional bifunctionality, PCL was mixed at 20 wt% with PCL–maleimide and templated into AAO from the 20 nm end and cooled to form J-HARPs within the template pores. 8-Mercaptoctylamine was then added to the 20 nm side of the AAO template to react with PCL–maleimide and selectively functionalize J-HARPs with amines only at the branched end that was exposed at the AAO surface (Figure 5a). We hypothesized that this fabrication process would lead to bifunctional J-HARPs with amine functionalities at the branched end and maleimide functionalities distributed throughout the remaining J-HARP backbone. To validate this approach, bifunctional J-HARPs were incubated with an IRDye-680 N-hydroxysuccinimide (NHS) ester to react with J-HARP amines followed by incubation with FITC-BSA to react with maleimides. We observed regional fluorescence of
dual-modified J-HARPs, with IRDye-680 observed only at the branched tips of the J-HARPs and FITC fluorescence observed throughout the remaining particle backbone (Figure 5b–d). In situ modification of J-HARPs within AAO templates therefore provides a straightforward method to fabricate particles with regional chemical handles for subsequent conjugation reactions and regiospecific functionalization.

3. Conclusion

The design and fabrication of polymeric particles with asymmetric Janus functionalities has been primarily limited to the fabrication of spherical particles using nonbiodegradable or specially synthesized polymers. Here we report a straightforward nanotemplating approach to fabricate high-aspect-ratio particles with Janus functionalities (J-HARPs) using biodegradable PCL polymers. J-HARPs were fabricated with branching morphologies at one edge of the particle, inspired by the morphologies and functionalities of high-aspect-ratio filamentous bacteriophage. Due to the increased surface area of the branching region of J-HARPs, we were able to achieve regioselective labeling and functionalization of J-HARPs with biomolecules such as proteins and biotin. Finally, we utilized in situ modification of J-HARPs within AAO templates to facilitate orthogonal bifunctionalities for dual labeling with regiospecific fluorescence. Taken together, these studies highlight J-HARPs as a new class of Janus materials with regional biofunctionalities that could see use in applications such as drug delivery and diagnostic medicine.

4. Experimental Section

General Methods and Instrumentation: Unless otherwise noted, all reagents were purchased from commercial sources. For the fabrication of J-HARPs, 10 kDa PCL was used. PCL—maleimide was prepared as previously described.\(^{[27]}\) PLGA (75:25 L:G, 35 000–45 000 Mn) and PLGA–PEG–biotin (50:50 L:G, Mₗ, 10 000 Da PLGA, 2000 Da PEG, PolysciTech) were used for the fabrication of biotinylated PLGA microparticles. J-HARPs were imaged using a Zeiss helium ion microscope at the University of California Berkeley Biomolecular Nanotechnology Center (Berkeley, California). Samples were sputter coated with gold–palladium at 10 mA for 45 s prior to imaging. All fluorescence microscopy studies were conducted at the UCSF Nikon Imaging Center using a Nikon Ti spinning-disk confocal microscope for cell studies and a Nikon 6D epifluorescence microscope for J-HARP bioconjugation studies.

J-HARP Fabrication: J-HARPs were fabricated by adapting a previously reported nanotemplating technique.\(^{[27]}\) A 100 mg mL\(^{-1}\) solution of 10 kDa PCL in trifluoroethanol was spun cast onto a glass wafer at 1000 rpm for 30 s, followed by heating at 150 °C to increase the homogeneity of the surface coating. After cooling, an AAO membrane (Anodisc 37 mm, 20 nm pore size) was placed atop the PCL film and transferred to a hot plate preheated to 60 °C. Templating occurred for 30 min, followed by removal and cooling of the AAO templates. The AAO templates were then etched in 5 M NaOH and shaken at room temperature for 25 min, followed by sonication in a bath sonicator for 10 min. The solution was then diluted twofold in ddH₂O and centrifuged at 3000 rpm for 10 min to cause J-HARP sedimentation. The NaOH supernatant was removed and the J-HARPs were washed two to three times with cold ddH₂O, followed by phosphate buffered saline (PBS), and finally washed two to four additional times with ddH₂O until a neutral pH was measured. The J-HARPs were then passed through a 40 μm cell strainer to remove large aggregates and stored in ddH₂O at 4 °C. To fabricate fluorescent J-HARPs, Nile red dye was added to the PCL/TFE solution at 0.5 mg mL\(^{-1}\) prior to spin casting and templating. PCL–maleimide was added to the PCL/TFE solution at 20 wt% relative to 10 kDa PCL prior to spin casting and templating.

Biotin Microparticle Fabrication: PLGA (75:25 L:G, 35 000–45 000 Mn) and PLGA–PEG–biotin were dissolved together in 2 mL dichloromethane (DCM) at 100 mg mL\(^{-1}\) PLGA and 10 mg mL\(^{-1}\) PLGA–PEG–biotin, followed by addition of DiR fluorophore (200 μL of 1 mg mL\(^{-1}\) solution in ethanol). 1 mL of 0.2% aqueous solution of poly(vinyl alcohol) (PVA) was then added to the polymer solution and sonicated using a probe sonicator for 25 s (5 s on, 10 s off; 30% amplitude), followed by addition of 6 mL 1% PVA. The solution was then transferred to a beaker and gently stirred while adding an additional 10 mL of 1% PVA. The solution was stirred for an additional 2–3 h at room temperature to allow for DCM evaporation. The particle solution was then spun down at 2000 rpm for 3 min to sediment the particles. PLGA microparticles were washed with several rounds of 1% PVA solution and lyophilized to yield 18.6 mg of fluorescent biotinylated microparticles.

J-HARP Modification and Functionalization: J-HARPs were suspended in a solution of 25 mM potassium phosphate buffer pH 7, to which was added an equal volume of 200 μmol solution of thiol-bearing biomolecules (FITC-BSA, biotin–PEG–thiol). The solution was mixed together and gently shaken at room temperature for 2 h. J-HARPs were then pelleted via centrifugation at 10 k rpm for 5 min and washed three to four times with PBS. For directional crosslinking experiments, a solution of biotin J-HARPs was added to an equal volume of 50 μM STV and incubated for 2–3 h prior to centrifugation and washing. STV J-HARPs were then added to an equal volume of biotinylated PLGA microparticles and mixed together while shaking overnight prior to imaging.

Cell Culture Studies: A549 lung cancer cells were cultured in F-12K media with l-glutamine, supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin, and incubated at 37 °C and 5% CO₂. HEK-293 cells were cultured in Dulbecco’s Modified Eagle Medium media with l-glutamine, supplemented with 10% FBS and penicillin/streptomycin. Cells were trypsinized, plated at 10 000 cells per well in 96-well plates, and incubated overnight. After overnight incubation, the medium was replaced with 100 μL medium containing J-HARPs or J-HARP controls and incubated for 6 h. The medium was then removed and cells were washed with PBS prior to imaging and/or fluorescence quantification. For imaging experiments, A549 lung cells were stained with CellMask Deep Red Plasmas membrane stain (ThermoFisher). To quantify J-HARP binding percentages, positive control wells of cells in PBS were doped with appropriate concentrations of J-HARPs, identical to the concentration of J-HARPs added initially to represent 100% binding efficiencies.

Statistical Analysis: For image analysis and quantification, 20 HARPs were selected from multiple images for gradient quantification. Imagej was used to evaluate fluorescence intensity across a 10 μm length of HARP, starting at one edge of the particle. Background fluorescence intensities were determined by measuring the fluorescence of an unoccupied region of the image, and backgrounds were subtracted from all data points. For each HARP analyzed, the maximal fluorescence data point was determined, and all other data points within that set were converted to % maximal fluorescence in relation to the highest fluorescence data point. % Max fluorescence data were then averaged, and data were plotted as mean ± standard deviation. Statistical significance was determined using a one-way ANOVA followed by Tukey’s multiple comparisons with Graphpad Prism software (p < 0.05, *p < 0.01).

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.

Data Availability Statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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bioconjugation, bioinspired materials, biotin, Janus, nanotechnology, particles, polymers

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