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Interrogating Cellular Functions with Designer Janus Particles†

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Abstract

Janus particles have two distinct surfaces or compartments. This enables novel applications that are impossible with homogeneous particles, ranging from the engineering of active colloidal metastructures to creating multimodal therapeutic materials. Recent years have witnessed a rapid development of novel Janus structures and exploration of their applications, particularly in the biomedical arena. It, therefore, becomes crucial to understand how Janus particles with surface or structural anisotropy might interact with biological systems and how such interactions may be exploited to manipulate biological responses. This perspective highlights recent studies that have employed Janus particles as novel toolsets to manipulate, measure, and understand cellular functions. Janus particles have been shown to have biological interactions different from uniform particles. Their surface anisotropy has been used to control the cell entry of synthetic particles, to spatially organize stimuli for the activation of immune cells, and to enable direct visualization and measurement of rotational dynamics of particles in living systems. The work included in this perspective showcases the significance of understanding the biological interactions of Janus particles and the tremendous potential of harnessing such interactions to advance the development of Janus structure-based biomaterials.

Graphical Abstract

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1. INTRODUCTION

Janus is the two-faced Roman god of beginnings and transitions, with one face looking to the future and the other to the past. Within the scientific community, the term Janus was first introduced by the late Nobel laureate Pierre-Gilles de Gennes to refer to particles that have different surface makeups on opposite hemispheres.¹ The first Janus beads, with one side hydrophobic and the other hydrophilic, were investigated in the 1980s.² Since then, research on Janus particles has blossomed far beyond the fields of colloids and soft matter. Much of the rapid progress has been made possible by the development of an expanding array of synthetic strategies for Janus particles.^{3–18} Today, Janus particles can be tailor-made in various sizes, shapes, and materials.^{3–10} They can be made amphiphilic, dipolar, or decorated with different biomolecules.^{11–14} Forms of surface anisotropy have expanded beyond half-and-half hemispheres to include interspersed small patches and other Janus geometries.^{7,15} Particles can be made by combining multiple building blocks of different chemical, optical, electrical, or magnetic properties.^{19,20} The synthesis of new types of Janus particles has opened up opportunities for many novel applications. One area of strong interest is the capacity of Janus particles to self-assemble into colloidal metastructures and the ability to engineer such structures.^{21–28}

For example, dipolar, amphiphilic, or magnetically responsive Janus particles can form unique crystalline and other ordered supracolloidal structures that would be virtually impossible to create with uniform particles. These metastructures alter their shape in response to environmental stimuli as small as a trace amount of ions and are thus a form of “active matter”.^{29–39}

Another fascinating research area explores the potential of Janus particles in biomedical applications, such as synergistic drug delivery, cell targeting, multimodal bioimaging, biosensing, and combinations of these capacities.^{14,40–51} Unlike uniform particles, Janus

particles allow diverse or incompatible functions to be combined in one structural entity. A single Janus particle with multiple compartments can serve as an ideal carrier for drugs of different water solubilities.⁵⁰ This, for example, offers a promising strategy for enhancing the efficacy of combination therapy, which requires the combined delivery of hydrophobic chemotherapeutic drugs and highly charged small interfering RNAs (siRNAs) that serve to prevent drug resistance. Encapsulated drugs can be released independently from separate compartments in a Janus particle made of polymers with different degradation properties.^{19,44,50,52–55} Many other sorts of otherwise incompatible functions, such as cell targeting, molecular sensing, and in vivo imaging, can also be combined into Janus particles.^{19,43,45,56} An early study has demonstrated that a cell-targeting function can be combined with a surface enhanced Raman spectroscopy (SERS) sensing capacity onto single Janus particles.⁴⁵ It is known that attaching molecules to surfaces of noble metals interferes with their SERS detection sensitivity. This problem was circumvented by attaching the cell-targeting ligands on one hemisphere of Janus particles and coating a roughened gold film on the other hemisphere for SERS sensing. Similarly, compartments of different magnetic and optical properties, such as magnetic and fluorescent nanoparticles, can be integrated to form Janus particles that enable simultaneous imaging, therapy, and diagnosis.^{42,43,45} In addition to all those exciting functions, Janus particles can also be rendered self-propulsive. When a gas-generating catalytic reaction occurs on one side of a particle, it propels the particle to move directionally. The self-propelled Janus motors have been demonstrated to be useful as active sensors and drug carriers.^{57–59} The examples mentioned here are only a glimpse of the vast variety of novel applications that have been made possible with Janus particles, but all demonstrate the tremendous potential for biomedical innovation promised by this novel particulate system.

While extensive studies have been focused on the capacity of Janus particles for biomedical applications, they are mostly about particle fabrication and proof-of-concept demonstration of their applications. Instead, an important but overlooked perspective is the understanding of the biological behavior of Janus particles. The interactions between Janus particles and biological systems must be carefully controlled to realize many of the promising biomedical applications. For example, Janus particles for multimodal cell-targeting must bind to cells selectively whereas ones for drug delivery must successfully cross the cell membrane to function inside cells. Yet we currently have little knowledge about how the surface or structural anisotropy of Janus particles might affect their interaction with biological systems. Simulation studies have predicted that when Janus particles interact with biological systems, their behavior deviates from that of uniform particles due to their surface anisotropy.^{60–62} We and other groups have also demonstrated this experimentally in a few recent studies.^{47,49,63–65} However, more studies from both theoretical and experimental perspectives are necessary to fully elucidate the role the anisotropy of Janus particles plays in their biological behavior. With such fundamental knowledge, we may then be able to rationally design Janus particle-based materials with desirable biological properties.

In this review, we highlight advances in understanding the interactions between Janus particles and cells and the use of such interactions as a novel means for interrogating various cellular functions. We summarize recent studies by our group and others that have employed the surface anisotropy of Janus particles to manipulate particle–cell interactions and cell

responses. In the first section, we will discuss how the anisotropic functionality of Janus particles changes the way by which they enter cells and how such basic understanding is translated into a new design strategy of drug delivery particles. The second section details studies of the use of Janus particles to achieve spatial and temporal control of the stimulation of immune cells. In the third section, we discuss research in which optically anisotropic Janus particles are employed for the quantitative measurement of dynamics in living cells that would otherwise be difficult, if not impossible, to probe with traditional methods.

2. CELLULAR UPTAKE OF JANUS PARTICLES

Controlling the uptake of particles by cells is a grand challenge for the development of therapeutic particles. Getting target cells to take up the therapeutic particles generally poses little difficulty—their binding to and internalization by target cells can be efficiently facilitated by specific ligand–receptor recognition. Instead, the major challenge is that the particles tend to be removed by the immune system before they ever reach their targeted tissues or cells. A subtype of immune cells, including macrophages and neutrophils, are specialized for detecting and ingesting pathogens, dead cells, and any foreign particles.^{66,67} This cellular uptake process is an essential part of the immune system's ability to fight against infections but is also responsible for the failure of many drug delivery particles, which are recognized as foreign.^{68,69} Extensive efforts have been devoted to developing strategies to control the immune cell uptake of synthetic particles, so that drug delivery particles can evade internalization by immune cells, while still be efficiently taken by their targeted cells. It is known that physical parameters of particles, including their size, shape, ligand density, and mechanical stiffness, play significant roles in influencing the cellular uptake of particles.^{70–91} For instance, immune cells most efficiently internalize particles that are 1–3 μm in diameter, a size range similar to most commonly found bacteria.^{83,92,93} For particles smaller than 2 μm , but not in other size ranges, the internalization efficiency increases with ligand density.⁷³ The surface chemistry of particles also influences their internalization by cells, as cationic particles generally penetrate cell membranes more efficiently than anionic ones⁹⁴ and immune cells prefer to uptake hydrophobic or highly charged particles over the neutral hydrophilic ones.^{79–83} Shape also exerts a significant influence over particle uptake, but studies have yet reached a consensus on which type of particle shape leads to the most efficient uptake. This is because the effect of particle shape appears to vary with other physiochemical properties of particles as well as the type of cells used.^{74,76–78,95–97} It was also reported that soft particles evade macrophage uptake more easily than hard ones.^{84–86} In spite of extensive studies, controlling the cellular uptake of synthetic particles remains a challenge.

Janus particles must function inside cells to achieve many biomedical applications. However, little has been done to understand how the anisotropic surface functionality of Janus particles affects their entry into cells or how it may be employed to control their cellular uptake fate. In a recent study, we demonstrated that the cellular uptake of synthetic particles can be controlled by spatial presentation of ligands on their surfaces.⁴⁹ We created Janus particles each of which displayed a patch of ligands and quantified how the cellular uptake probability of such particles depended on the size of the ligand patch. The Janus particles were created by using a microcontact printing method, in which a patch of bovine serum

albumin (BSA)–biotin was transferred from a polydimethylsiloxane (PDMS) elastomer onto one pole of silica particles (Figure 1a). The ligand immunoglobulin G (IgG) was then conjugated to BSA–biotin via streptavidin linkers, and the exposed surface of the particles was passivated by BSA, which does not initiate particle uptake by macrophage cells. Using fluorescence microscopy, we measured the ligand patch size (characterized as the arc angle of each patch) of single Janus particles and determined whether each particle was inside of a cell or outside. By making this quantification for a population of hundreds of such particles, we determined the fraction of internalized particles as a function of ligand patch size. The cells used were macrophages, a type of immune cells responsible for removing foreign particles. We found that, for both 1.6 and 3 μm particles, the probability that particles are internalized by macrophage cells increases with the ligand patch size (Figure 1b). Interestingly, we also found that Janus particles with a patch of ligands are less likely to be internalized than particles that are uniformly coated with the same number of ligands. This is a first study to demonstrate that a partial coating of ligands on particles effectively reduces their cellular uptake by macrophage cells.

We next sought to explore how our findings from this initial study might be used to change the design of drug delivery particles. Synthetic particles designed as drug carriers are generally coated with targeting ligands and a dense layer of polyethylene glycol (PEG).^{98–100} The PEG coating prevents antibodies and blood serum proteins from being nonspecifically adsorbed onto particles as they circulate in the bloodstream. This, in turn, prevents immune cells from detecting the particles as foreign and removing them.^{101–105} While the PEG coating is effective in protecting drug delivery particles from immune detection, the bulky PEG chains also sterically hinder the efficient binding of the particles to their targeted cells.^{106–109} Our study, as mentioned above, showed that a partial coating of IgG antibody reduces the fraction of particles cleared by immune cells. We therefore hypothesized that separating PEGs and targeting ligands on the two hemispheres of single Janus particles can combine these two functions on the same particle while reducing their interference with one another.⁶⁵

As our first step toward testing this hypothesis, we determined whether a half coating of PEGs is effective in reducing the immune clearance of particles. To create half-PEGylated Janus particles, we conjugated thiol-PEG_{5k} onto the gold hemisphere of Janus particles at a surface density of 0.08 ± 0.02 PEG per nm^2 (Figure 2a). PEG chains at such grafting density exhibit the “brush” conformation, which is required for them to effectively prevent adsorption of proteins. The PEGylated hemisphere of Janus particles was found to exhibit negligible adsorption of IgG antibodies, while the non-PEGylated hemisphere showed a significant accumulation of IgG (Figure 2b). We found that, despite the adsorption of IgG onto one hemisphere, the half-PEGylated Janus particles were internalized by macrophage cells at a probability nearly as low as that of particles with a full PEG coating (Figure 2c). The half coating of PEG reduced the immune cell internalization probability to $\approx 20\%$, by comparison with $\approx 70\text{--}80\%$ for particles with no protective PEG layer at all. This phenomenon held for particles of 500 nm, 1.2 μm , and 1.6 μm in diameter. A half coating of PEGs is therefore effective in preventing immune clearance of particles. We next incorporated targeting ligands onto the other hemisphere of the PEGylated Janus particles. The targeting ligand we chose was anti-CD3, an antibody that binds T cell receptors on

Jurkat T cells. While particles with a uniform coating of PEG and anti-CD3 bound to T cells at a low probability ($4.2 \pm 0.7\%$) as expected, Janus particles functionalized with PEG on one side and anti-CD3 on the other exhibited a significantly higher cell targeting probability ($26.4 \pm 0.8\%$) (Figure 2d). This comparison highlights the potential of therapeutic particle designs that include a spatial separation of PEGs and cell-targeting ligands. Such a design could reduce the interference between these two functions while maintaining the protective effect of PEGs against immune cell internalization. However, the effectiveness of this strategy must be further evaluated with nanoparticles that are 100 nm or smaller, because this is the common size range of drug delivery particles. It must also be tested in vivo as well as with different cell types. The work done so far has opened a door to many possible studies to explore how the surface anisotropy of Janus particles can be used to advance the design of therapeutic particles.

To understand why the anisotropic presentation of ligands or PEGs reduces the cellular uptake of particles, we investigated the cellular entry mechanisms of Janus particles and the role of surface anisotropy in the process.^{47,65} We compared the protrusion of the cell membrane around half-coated Janus particles with that observed for uniformly coated particles. For a uniformly coated particle, engulfment occurs by processive protrusion, which eventually results in closure of the cell membrane over the particle. The protrusion of the cell membrane around half-coated Janus particles was altered by the spatial arrangement of ligands and occurred through a three-stage process (Figure 3a,b). The cell membrane first quickly wrapped around the ligand-coated hemisphere, stalled at the Janus interface, and then eventually engulfed the noncoated side of the Janus particles. We confirmed that the morphology of cell membrane protrusion differs on the two hemispheres of Janus particles by resolving the membrane structure using scanning and transmission electron microscopies (SEM and TEM, respectively) (Figure 3c). On the ligand-coated hemisphere, the cell membrane wrapped tightly around particles, as expected from ligand–receptor binding. In contrast, large cell membrane ruffles were observed on the hemisphere without ligands, whether it was passivated with BSA or coated with a dense layer of PEGs. Inhibition of membrane ruffling reduced the fraction of half-coated Janus particles that were internalized. Together, the results demonstrated that the mechanism of cell entry differed on the two hemispheres of half-coated Janus particles. Ligands on one hemisphere of the Janus particles initiated and guided the membrane wrapping around the particles, whereas the nonligand hemisphere was completely engulfed by large membrane ruffles that occur without ligand–receptor binding. Owing to the asymmetric presentation of ligands, a half-coated Janus particle requires a combination of both mechanisms in order to enter cells. Because not all membrane ruffling events lead to successful engulfment of particles, some Janus particles fail to enter cells. This explains why the internalization probability for half-coated Janus particles was reduced in comparison to uniformly coated ones. It is known that synthetic particles enter cells mostly via ligand-guided pathways and occasionally via a so-called “cell drinking” process enabled by the large cell membrane ruffles.^{110,111} However, our studies demonstrated experimentally that those different cell entry mechanisms could be integrated into a single cell entry event. Much effort has been devoted to controlling the cellular entry pathways of therapeutic particles, because particles entering cells via different pathways may reach different organelles inside cells and be degraded differently.^{70,112} Our studies

suggest that the surface anisotropy of particles may be a new parameter one can use to manipulate the cellular entry pathway of particles.

3. JANUS PARTICLES FOR MANIPULATING IMMUNE CELL STIMULATION

Inspired by the ways that cells communicate in the body, the development of cell-mimicking particles for therapeutic applications is rapidly emerging. Synthetic particles that mimic natural cells in size, shape, mechanical stiffness, and the way molecules are presented on their surfaces have shown unique advantages when compared with particles that do not exhibit those physiochemical characteristics.^{113–115} Among the many promising applications of cell-mimicking particles, the development of particles as artificial antigen-presenting cells for in vitro stimulation of T cells is of particular interest. This has direct application in an emerging cancer treatment called adoptive T cell therapy. In this approach, a patient's own T cells are restimulated in culture flasks and injected back into the patient's body to fight against tumors. In contrast to traditional cancer treatments such as chemotherapy or bone marrow transplants, adoptive T cell therapy can potentially reduce the risk of immune rejection and give cancer patients a longer-lasting remission.^{116–120} But its broad clinical application is hindered by the technically demanding procedure needed to generate large numbers of antitumor T cells for each patient.^{121–123} Inside a human body, efficient activation and proliferation of T cells requires interactions with antigen-presentation cells (APCs) and, in particular, with dendritic cells. Unfortunately, the preparation of native APCs for clinical use is a challenging task. Isolation and in vitro stimulation of APCs is time-consuming and expensive; the quality of patient-derived native APCs is also highly variable due to potential suppression by the tumor microenvironment, which may be responsible for the inconsistent results observed in clinical trials.^{116–118,124} To overcome these difficulties, artificial antigen-presenting cells (aAPCs) made from synthetic materials have been developed as an alternative method for stimulating T cells in vitro.

The simplest form of aAPCs consists of spherical particles functionalized with antibodies or antigens for T cell stimulation.^{125–128} Additional costimulatory or inhibitory ligands can either be coated on the surfaces of particles or encapsulated inside them for delivery on site. While such simple aAPC particles provide consistent signals for T cell stimulation and are easy to manufacture, they do not interact with T cells in the same way as a natural APC does. As a result, in many cases they have shown limited efficacy in sustaining long-term growth of T cells.¹²⁹ Therefore, there is an emerging interest in developing aAPC particles that not only display the necessary ligands for T cell stimulation but also mimic key physiochemical properties of natural APCs. For example, aAPCs based on ellipsoidal particles,¹³⁰ carbon nanotubes,^{131,132} and polymer "nanoworms"¹³³ have shown enhanced multivalent binding to T cells and have improved the efficacy in T cell stimulation. One important characteristic of the interactions between a natural APC and a T cell is the formation of a protein pattern in the interface between the two cells.^{134,135} When T cells are activated, the ligand-bound receptors form clusters and segregate into a "bull's eye" pattern with several distinct concentric domains. Ligand-bound T cell receptors (TCRs) accumulate in the center while integrins are enriched in a surrounding ring structure (Figure 4a). The

formation of this protein pattern is critical for T cell activation,^{136–141} but the importance of replicating it in the design of aAPC particles had not been explored.

We created the first Janus particle-based aAPCs displaying “bull’s eye” protein patterns and investigated their efficacy in stimulating T cells in vitro.⁴⁶ Our bifunctional Janus particles were functionalized with anti-CD3 antibodies that bind TCRs and fibronectin molecules that bind $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrins for T cell adhesion. We created two different types of “bull’s eye” particles in order to determine the effect of these ligand patterns on T cell activation (Figure 4b). One type had a pattern resembling the natural protein pattern, with anti-CD3 concentrated in the central domain and fibronectin in the surrounding region. The other protein pattern is the reverse type, with a patch of fibronectin surrounded by a field of anti-CD3. Both types of “bull’s eye” particles were fabricated using the same microcontact printing method with alternating proteins as “ink”, as illustrated in Figure 4c. The activation of Jurkat T cells was quantified by measuring the intracellular calcium flux. T cell activation causes an influx of calcium ions, and the stronger the T cell activation, the more intense and sustained the calcium influx.^{142,143} The amplitude and duration of calcium elevation in T cells therefore serve as a direct indicator of the strength of T cell activation. The calcium influx was measured using the fluorescence intensity of a calcium-sensitive dye, Fluo-4, whose fluorescence intensity increases in proportion to the cytosolic concentration of calcium ions. We found that particles with the reverse “bull’s eye” pattern activate T cells better than ones with the native protein pattern, in that they activate a larger fraction of T cells and lead to more intense and sustaining calcium responses (Figure 4f–h). We also demonstrated that the different patterns of ligands on the aAPC particles dictate the spatial arrangement of intracellular signaling proteins, including protein kinase C (PKC)- θ and actin, in T cells. Because the subcellular location of proteins directly regulates their signaling functions, the reorganization of PKC- θ and actin explains why the ligand patterns on aAPC particles change the overall T cell activation response. Our observation that the reverse “bull’s eye” protein pattern produces the stronger T cell activation also agrees with previous reports that the central accumulation of TCRs leads to signaling termination.^{135,144} Previous studies have shown that T cell activation can be sustained if the central accumulation of TCRs is delayed or disrupted. This is the case for the T cell activation by the reverse “bull’s eye” particles. Our results demonstrate that the in vitro stimulation of T cells can be manipulated by simply changing the organization of ligands in the “bull’s eye” pattern. This proof-of-concept study shows how anisotropic surface functionality of Janus particles can be exploited to fine-tune T cell activation with potential applications in adoptive T cell therapy.

The main limitation we encountered in this proof-of-concept T cell activation study was the lack of control over contact between particles and T cells. Because the “bull’s eye” pattern was on only one hemisphere of the Janus particles, T cell activation depended on the orientation of the particle with respect to the cell. We next sought to achieve spatial and temporal control over T cell activation by aAPC particles. Researchers typically control interactions between particles and cells using one of two forms of external stimuli: light or magnetic force. The optical tweezers, which move particles using light, have been widely used to manipulate cell behaviors. But the application of optical manipulation is impeded by the limited penetration depth of light into tissues¹⁴⁵ and by its substantial phototoxicity to

cells.^{146–148} Magnetic techniques eliminate such concerns. In some prior studies, a rotating magnetic field was applied to twist magnetic nanoparticles. Researchers have used such manipulations to control the opening of ion channels,¹⁴⁹ measure the mechanical properties of the cytoplasm and cytoskeleton of the cell,^{150,151} and probe mechanotransduction in cell surface receptors.¹⁵² In some other studies, the aggregation of magnetic nanoparticles under an external field has been used to cause cell surface receptors to cluster to produce controlled apoptosis of tumor cells^{153–155} or to regulate the nucleation and assembly of cytoskeleton proteins inside cells.¹⁵⁶ In those studies, the twisting or aggregation of magnetic nanoparticles cannot be controlled spatially or on a single-cell level, unless complex devices, such as magnetic tweezers, are used.

We chose to use magnetic fields to remotely control the interactions between particles and T cells. Instead of complex magnetic devices, control was achieved using simple hand-held magnets by taking advantage of the properties of magnetically responsive Janus particles.¹⁵⁷ Silica particles were coated with a thin nickel coating on one hemisphere followed by an aluminum coating to protect the nickel from oxidation. This half-coating of nickel results in a net magnetic dipole parallel to the Janus interface and shifted away from the geometric center of the particles.^{9,158} Due to the positioning of the magnetic dipole, the rotation and locomotion of single Janus particles can be controlled by a rotating magnetic field generated from a hand-held magnet. Janus particles of either spherical or rod-like shapes can be controlled in the same manner (Figure 5a–c). To activate T cells, we functionalized the exposed silica hemisphere with the stimulatory ligand anti-CD3 antibody. This ligand-coated hemisphere serves as the “on” switch of T cell activation. Its initial contact with a T cell, which is controlled by the magnetic field, determines the initiation of T cell activation (Figure 5d). To activate a single T cell, we first moved the Janus particle to make contact with the cell from its metal-coated hemisphere and then rotated it so that its ligand-coated side triggered T cell activation. As mentioned above, we used the elevation of calcium concentration in the cytosol, which was measured by using the calcium-sensitive dye Fluo-4, as a direct indicator of the T cell activation. Every cell observed ($N > 50$) became activated following the binding of the ligand-coated side of the Janus particle to the cell, and the activation persisted for over a minute before gradually decreasing to the basal level (Figure 5e). The onset of T cell activation was controlled by the timing of the rotation of the anti-CD3 side to face the cell (Figure 5f). By controlling the lateral trajectory of a Janus particle, we could choose which individual cell to stimulate. In addition to spherical Janus particles, this approach was demonstrated with rod-shaped magnetic Janus particles. This study demonstrates that the surface anisotropy of Janus particles may be used as a remote switch for controlling the initiation of cell responses. Overall, our aforementioned studies showcase a new application of Janus particles for the spatial and temporal manipulation of cell stimulation.

4. JANUS PARTICLES AS OPTICALLY ANISOTROPIC PROBES FOR MEASURING ROTATIONAL DYNAMICS IN LIVING SYSTEMS

The functions of biological systems involve cascades of dynamic events, from diffusion of enzymes, to trafficking of intracellular organelles, to migration of whole cells. These

dynamics are typically depicted in terms of translational motion, in part because it is straightforward to measure. By following the movement of the objects of interest, whether they are molecules, viruses, cells, or particles, one can uncover transient interactions that are essential for cell functions.^{159–161} However, it is becoming more evident that rotational dynamics are also abundant in living systems. For example, kinesin and myosin molecular motors rotate as they “walk” along microtubules or actin filaments.^{162,163} To understand the complex interactions that give rise to those rotational dynamics, it requires a way of measuring them. Tracking rotational dynamics is substantially more challenging than tracking the translational motion of single objects. Only a handful of techniques have been developed to measure the rotation of single particles; all rely on the use of probes that are either optically or geometrically anisotropic. Geometrically anisotropic probes include rod-like viruses,¹⁶⁴ rod-shaped colloidal clusters,¹⁶⁵ ellipsoidal polymeric particles,¹⁶⁶ nanocrystals,¹⁶⁷ quantum rods,¹⁶⁸ and gold nanorods.^{169–172} Methods for tracking the rotation of such single-particle probes have been summarized in our previous review.¹⁷³

The optically anisotropic probes used for imaging single particle rotation can generally be categorized as Janus particles, even though some were not classified as such when they were reported. One way of introducing optical anisotropy to an isotropic particle is to attach a fluorescent nanoparticle to the surface of the particle of interest. Such a technique has been used to measure the longitudinal rotation of a Gram-positive bacterium *Listeria monocytogene*¹⁷⁴ and the rotation of single viruses upon binding to viral receptors on a lipid membrane.¹⁷⁵ In the latter study, a quantum dot was attached to the capsid of each Simian virus 40. The angle and distance between each virus and its surface-tethered quantum dot, which were imaged using interferometric scattering and fluorescence microscopy, respectively, allowed the calculation of the orientation of the virus in three dimensions. This straightforward method is generally useful for tracking the rotation of nanosized particles ranging from viruses to synthetic particles. But the technique is limited by the fact that the number of surface-tethered nanoprobe per particle cannot be controlled using current conjugation methods. Because of this, some particles may have too many nanoprobe to be useful for accurate imaging and tracking of the particle rotation. The surface attachment of nanoprobe also changes the original geometry of the particle of interest, which may bias its rotation. A second approach to creating optically anisotropic Janus particles is to coat one hemisphere of the fluorescent particles with metal. The metal cap blocks the fluorescence from one side of the particles, so the particles appear fluorescent on one side and dark on the other in imaging, much as the moon is dark on one side and light on the other due to the illumination of the sun. To reflect the distinctive appearance of these Janus particles, researchers named them modulated optical nanoprobe (MOONs).^{176,177} As the MOONs rotate, the position and the overall intensity of the bright hemisphere change with particle orientation in a manner similar to the phases of the moon. This allows particle rotation to be measured.¹⁷⁸ The rotation of MOONs has been used to measure the local rheological properties of solutions,^{179,180} the translational–rotational coupling of condensed colloids,^{181,182} and the rotational dynamics of two-dimensional colloidal crystals.¹⁸³ Because measuring one of the rotational angles precisely requires resolving the crescent shape of the fluorescence hemisphere, this method is so far limited to micrometer-sized MOONs. The large size of the particle probes may limit their application in biological systems.

We recently reported a different way to measure the rotation of single particles.¹⁸⁴ Janus particles were made to display two patches of BSA, labeled with either Alexa 488 or 568 dyes, on opposite poles (Figure 6a,b). This optical anisotropy allows the rotation of single particles to be directly visualized and measured. Custom image processing algorithms were developed to locate the centroid of each patch and draw a vector between the centroids of the two patches belonging to a single particle. The orientation of the vector indicates the in-plane orientation of the particle, and the center of the vector corresponds to the geometric center of the particle (Figure 6b). We used this method to investigate the rotational dynamics of particles during their entry into macrophage cells. The results revealed a heterogeneous mixture of rotational movements of particles during this process, each corresponding to a different stage of the interaction between the particle and the cell (Figure 6c). Particles underwent rapid directional rotation when they first made contact with cells, reflecting a process during which cells actively search for ligands and pull particles toward the cell body. Particle rotation slowed down and appeared as random “jiggling” once the particles were attached to the cell body ready for cell entry. Rapid directional rotation was observed again after the complete engulfment of the particles, suggesting active rotation of the endosomes inside cells. The measurement of rotation provides a new dimension of information, in addition to what can be extracted from translational motion, about particle–cell interactions. While we reported measurement of only the in-plane rotational angle of the Janus particles in our first study, it is possible to also obtain the rotational angles along other axes using this method. Progress is ongoing in our laboratory. Unlike the single-particle rotation tracking method that uses MOONs, our tracking method is technically applicable to nanoparticles smaller than 500 nm, as long as two discrete patches can be made on a single particle.

5. CONCLUSION AND OUTLOOK

Janus particles have emerged as an exciting new type of particulate material. They consist of multiple building blocks that differ in their chemical, physical, or material properties. Such surface and structural anisotropy allows multiple functions to be combined into a single particle and thus enables a diverse range of novel applications that could not be achieved using conventional particles with uniform composition. Studies to date have demonstrated the tremendous potential of Janus particles for a wide range of applications from active matter engineering to biomedical innovations. By comparison, relatively little has been done to explore the fundamental aspect of Janus particle research, such as interaction of particles with biological systems and the role that surface or structural anisotropy plays in such interactions. Here, we have highlighted this less known side of the story. The work summarized here illustrates advances in understanding the biological behavior of Janus particles and how the surface anisotropy of Janus particles can be employed to manipulate, measure, and understand cellular functions. Three different types of examples were discussed. For the function of cellular entry, Janus particles with a partial surface coating of ligands were shown to enter cells differently than uniformly coated particles. This provides a new way to control the cellular uptake of synthetic particles, which is crucial for exercising control over the fate and efficacy of drug delivery particles. This fundamental knowledge has led to the development of a spatial decoupling strategy for dual-functional Janus particles. Janus particles can be coated with PEGs to evade uptake by the immune system on one

hemisphere and with ligands for targeting a particular type of cell on the other. This allows a simple particle to perform both these functions while minimizing their interference with one another. The potential for using Janus particles to manipulate the responses of cells was further demonstrated in the context of immune cell activation. Janus particles whose anisotropic presentation of ligands mimics the protein arrangement on natural antigen-presenting cells were used to activate T cells. Different patterns of ligands on particles were found to lead to different levels of immune activation response. Moreover, the remote control of the orientation and locomotion of magnetic Janus particles can be used as a switch to temporally control the onset of the immune cell activation. Using the surface anisotropy of Janus particles in yet another way, such particles were made optically anisotropic to allow direct visualization and measurement of rotational dynamics occurring in living cells. The rotation of particles reveals a new dimension of information about biological interactions that remains hidden in conventional measurements of translational motion.

As more types of Janus particles are synthesized and their novel applications are explored, understanding the interactions between Janus particles and biological systems will provide us with the predictive ability to design Janus particle-based materials with defined functions and properties. Such fundamental understanding is also essential to properly assess the potential impacts of Janus particle-based materials on living systems. The studies summarized here represent no more than the beginning of such effort. The discussion here is intended to bring attention to this currently overlooked area so that more thorough investigation will be done to elucidate the general principles and mechanisms governing the biological behavior of Janus particles. This fundamental understanding will in turn guide the rational design of Janus particles for biomedical applications.

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Figure 1.

Cellular uptake probability (% internalization) of Janus particles by macrophage cells depends on the size of the ligand patch. (a) Schematic illustration of the microcontact printing method for fabricating Janus particles displaying a patch of ligands. A PDMS stamp preinked with BSA–biotin is pressed against a monolayer of particles and then peeled off. Janus particles are removed from the PDMS stamp by sonication. Subsequently the BSA–biotin patch on the Janus particles was functionalized with rabbit IgG via biotin–streptavidin binding. (b) Uptake probability of the 1.6- μm Janus particles ($\blacktriangle$) plotted against the arc angle θ of the IgG patch and that of uniform all-IgG particles (O) plotted against the total number of IgG on the particle surface. Each data point is an average of >40 particles. Error bars represent standard deviations between at least two independent samples. Inset is a schematic illustration of the angle θ . Arc angle θ characterizes the size of the ligand patch and is defined as the angle subtended by this longest dimension of a protein patch on the Janus particle surface. Reproduced from ref 49 with permission. Copyright 2015 The American Chemical Society.

Figure 2.

(a) Schematic illustration of the fabrication procedure of half-PEGylated and fully PEGylated particles. (b) Bright-field and fluorescence images showing three types of Janus particles as indicated, and the nonspecifically adsorbed IgG-Alexa488 (fluorescent). The fluorescence intensity of IgG is shown on the same color scale in all three images to highlight the difference in intensity. All particles are 1.6 μm in diameter. (c) Internalization probability of three types of particles (after adsorption of IgG) by macrophage cells. The 500 nm and 1.2 μm all-PEG and half-PEG Janus particles were statistically similar at the $p < 0.05$ level. The internalizations of 1.6 μm all-PEG and half-PEG Janus particles were statistically different at the $p < 0.05$ level. Results for each type of particle were from 25 to 48 images in 2–6 independent samples. (d) The binding probability of 500 nm particles to T cells. The fraction of membrane-bound particles is $26.4 \pm 0.8\%$ for PEG-Anti-CD3 Janus particles and $4.2 \pm 0.7\%$ for particles uniformly coated with PEGs and anti-CD3 (PEG-Anti-CD3 uniform). A total of $N = 258$ T cells were analyzed for the PEG-Anti-CD3 Janus particles and $N = 82$ cells for the uniformly coated particles. Inset demonstrates the unidirectional binding of the Anti-CD3 coated hemisphere (shown in red) of 1.6 μm Janus particles to Jurkat T cells (shown in green). The nonfluorescent hemisphere of the Janus particles was coated with PEGs. The images are representative of the entire population of 16 particles and 12 cells. Scale bars in all images are 3 μm . Reproduced from ref 65 with permission. Copyright 2017 The Royal Society of Chemistry.

Figure 3.

Cellular entry mechanism of half-coated Janus particles. (a) Overlay confocal fluorescence images of the cell membrane (green) and the ligand-coated hemisphere (red) of a Janus particle at different elapsed times as indicated. (b) Four angles, α , β , α' , and β' are defined (inset). In all cross-sectional images, α and β measure the angles from either rim of the membrane cup to the initial particle-cell contact point. α' and β' measure the angles from either Janus interface to the initial particle-cell contact point. α and β obtained from the Janus particle shown in (a) are plotted against time. The plateau region of β is indicated by the shaded gray area. Dotted lines indicate α' and β' , which characterize the orientation of the Janus particle. The observation is representative of >40 internalization events. A schematic illustration (c), pseudocolored SEM images (d), and TEM images (e) demonstrate the different membrane morphologies on the two hemispheres of IgG-PEG Janus particles. In the pseudocolored SEM images, the PEGylated hemispheres are shown in purple and the IgG-coated hemispheres are shown in green. Scale bars: 5 μm in (a), 1 μm in SEM images (d) and 500 nm in TEM images (e). Reproduced from refs 47 and 65 with permission.

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Figure 4.

Activation of Jurkat T cells by "bull's eye" Janus particles. (a) Schematic illustration of the formation of protein pattern in the membrane junction between a T cell and an antigen-presenting cell. Ligand-bound T cell receptors (TCRs) and integrins form "bull's eye" concentric microdomains. (b) Schematic illustration of Janus particles that display patterns of anti-CD3 and fibronectin that mimic either the native or reverse "bull's eye" pattern. (c, d) Reconstructed 3-D confocal fluorescence images of the "bull's eye" particles. (e) Schematic illustration of the microcontact printing method for creating "bull's eye" Janus particles. (f, g) Jurkat T cells were loaded with calcium-sensitive dye, Fluo-4 AM, whose fluorescence intensity increases with intracellular concentration of Ca^{2+} . In the two plots shown, normalized fluorescence intensities of T cells stimulated by the reverse (total number of cells: 96) and the native "bull's eye" particles (total number of cells: 111) are plotted on a color scale and sorted based upon the fluorescence intensity of the first peak. (h–j) Calcium response of T cells that are in contact with the "bull's eye" patterns was quantified with three parameters: the fraction of activated T cells (% activation), activation intensity (average fluorescence amplitude), and duration of calcium response (response fraction). Average fluorescence amplitude and response fraction are plotted in scattered plot with the mean $\pm$ SEM (shown in red). For the native pattern, a total of 30 cells were analyzed, and for the reverse pattern, 24 cells. Either data set represents results from more than 10 experiments done over 4 different days. Scale bars: 5 μ m. Reproduced from ref 46 with permission. Copyright 2014 The American Chemical Society.

Figure 5.

Spatiotemporal control of T cell activation using magnetic Janus particles. (a) A schematic showing experimental setup used to apply magnetic field to Janus particles. Janus particles were controlled by rotating magnetic field generated by the rotation of permanent magnets. (b) Superimposed bright-field images showing that rotation and locomotion of a single Janus sphere can be simultaneously controlled to circumvent stationary particles (indicated by the yellow arrows) that were not magnetically responsive. (c) Superimposed bright-field images showing rotation and locomotion control of a Janus rod. (d) Schematic illustration of the way T cell activation was controlled by the rotation and locomotion of magnetic Janus particles. (e) Fluorescence images showing T cell activation when the anti-CD3 coated hemisphere of a Janus sphere (indicated by the yellow arrow) was rotated to face the cell. Images are color-coded based on fluorescence intensity of the calcium reporter Fluo-4. (f) The normalized fluorescence intensity of the T cell shown in (e) is plotted against time to show the time dependence of T cell activation. The red arrow indicates the time when the metal-coated hemisphere made contact with the T cell, and the blue arrow indicates the time when the anti-CD3 side was rotated to face the cell. Scale bars: 5 μm in (b) and (c), and 10 μm in (e). Results in (e) and (f) are representative of 51 cells. Reproduced from ref 157 with permission. Copyright 2016 Wiley-VCH Verlag GmbH & Co.

Figure 6.

Dual-color Janus particles as probes for measuring single particle rotation. (a) Schematic illustration of the “sandwich” microcontact printing method for creating the dual-color Janus particles. The proteins to be printed on the particles are illustrated in red and green. A protein incubation step follows the microcontact printing to coat the particles either with BSA for passivation or with BSA–biotin for uniform ligand coating. (b) Single-particle tracking of the dual-color Janus particles with a vector pointing from the green to the red patch. The numbers are arbitrarily assigned IDs for particles. Inset is a magnified image of particle 3. Scale bar: 5 μm . (c) In-plane rotational angle θ of a single Janus particle as it bound to and entered a macrophage cell is plotted as a function of time. The different stages of interactions, particle-cell binding, cell entry, and dynamics inside cells, are indicated in gray, red, and blue shades, respectively. Reproduced from ref 184 with permission. Copyright 2015 The Royal Society of Chemistry.