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Surface Functionalization of Gold Nanoparticles with Red Blood Cell Membranes

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Gold nanoparticles (AuNPs) find widespread applications as imaging agents and drug carriers in biology and medicine owing to their biocompatibility, optical properties, and easily modifiable surfaces.^[1, 2] A myriad of molecules including drugs,^[3] siRNA,^[4] DNA,^[5] and proteins^[6] have been coupled with AuNPs for a large spectrum of biomedical applications ranging from *in vitro* biosensing to *in vivo* drug/gene delivery. The facile coupling process between gold and thiol also enables various stabilization and stealth strategies that enhance AuNPs' utility. Through the use of thiolated ligands, biocompatible polymers such as polyethylene glycol^[7, 8] and zwitterionic polymers^[9, 10] have been conjugated to AuNPs to improve their *in vivo* stability and prolong their systemic circulation lifetime. These long-circulating AuNPs offer the capability to preferentially accumulate at tumor sites through a passive or active targeting mechanism and enter cells through endocytosis. In addition to thiolated ligands, other strategies have also been explored to minimize the risk of opsonization and potential ligand displacement.^[11] Among the many functionalization methods, lipid membrane coating presents a versatile approach that enables both surface shielding and functional ligand incorporation. Through phospholipid coatings, AuNPs can be insulated from external environment by a layer of hydrophobic acyl chains and yet remain readily modifiable with lipid-tethered ligands.^[12] Emerging bio-inspired functionalization strategies have also stemmed from lipid-membrane-coated AuNPs, which have been used to construct high density lipoprotein (HDL)-mimicking nanoparticles via the spontaneous incorporation of apolipoprotein into the lipid membranes.^[13] Toward engineering AuNPs with improved functions and advanced biomimetic features, herein we report a new and elegant strategy in AuNP modification using the entire functional utility of a cell membrane, red blood cell (RBC) membrane in particular.

RBCs are nature's long-circulating delivery vehicles that continually inspire the design and engineering of artificial biomaterials and delivery systems.^[14] It has been found that the

recognition of homologous RBCs as self is mediated by a variety of proteins residing on the cell membranes.^[14] For example, CD47 has been identified on RBC surfaces as a self-marker that actively signals macrophages and prevents their uptake.^[15, 16] Other membrane proteins including C8-binding protein (C8bp),^[17] homologous restriction protein (HRP),^[18] decay-accelerating factor (DAF), membrane cofactor protein (MCP), complement receptor 1 (CR1), and CD59^[19] on RBC surfaces fend off the attack by the complement system. It is expected that nanoparticles possessing these surface properties of RBCs should be less susceptible to clearance by phagocytic cells. Driven by the concept of bridging synthetic and natural biomaterials for nanoparticle functionalization and by our recent success in translocating intact membranes and peripheral proteins from RBCs to the exterior of soft colloidal particles made of poly(lactic-co-glycolic acid),^[20] this work aims to apply the novel RBC membrane coating technique to inorganic AuNPs. We demonstrate that RBC membrane coating effectively shields AuNP surfaces from thiolated probes and significantly reduces phagocytic uptake of the particles.

As illustrated in Figure 1, the process of functionalizing AuNPs with natural RBC membranes consists of two steps: deriving membrane vesicles from RBCs and fusing the vesicles onto the surfaces of AuNPs. In the study, purified mouse RBCs underwent a hypotonic treatment to remove their intracellular contents. The resulting RBC ghosts were then extruded through 100-nm porous membranes to generate RBC-membrane-derived-vesicles with a hydrodynamic diameter of approximately 100 nm.^[20] To examine the coating of RBC membranes onto AuNPs, citrate-stabilized AuNPs with a diameter of 70 nm were mixed with the membrane vesicles and subsequently extruded through 100-nm pores. The mechanical force provided by the extrusion process facilitated the fusion of RBC membrane vesicles with solid AuNPs, thus forming RBC-membrane-coated AuNPs (RBC-AuNPs). Repeated passing through the extruder was performed to ensure complete particle coating with a homogeneous lipid shell.^[21]

The RBC membrane coating on AuNPs was first monitored by the changes in AuNP size and surface charge before and after the fusion process (Figure 2A). Following membrane fusion, the diameter of the AuNPs increased from 70.1 ± 0.3 nm to 86.5 ± 0.2 nm, which can be the result of an 8 nm-thick lipid shell corresponding to the membrane width of RBCs.^[22] The surface zeta potential of AuNPs also changed from -42.2 ± 1.3 mV to -35.1 ± 1.1 mV upon fusing with the RBC membrane vesicles. This shift was likely due to the charge screening by RBC membranes, as RBCs under different conditions are found to possess a less negative surface charge as compared to citrate-stabilized AuNPs.^[23] To further confirm the RBC membrane coating, AuNPs were stained with uranyl acetate and visualized by transmission electron microscopy (TEM). Figure 2B shows that all particles had a spherical, core-shell structure that reflected the enclosure of AuNP in a thin shell. Collectively, these results demonstrate the successful coating of AuNPs with unilamellar RBC membranes. Moreover, the resulting RBC-AuNPs were stable in both PBS buffer solution and 100% fetal bovine serum; no detectable increase in particle size was observed over a span of 72 hrs (Figure 2C).

To further characterize the RBC-AuNPs, we examined the particles' interaction with thiolated ligands using a fluorescein isothiocyanate (FITC)-thiol conjugate. The thiolated

fluorescent probe was prepared by first conjugating FITC to cysteamine 4-methoxytrityl resin through NHS-amine coupling, followed by trifluoroacetic acid (TFA) treatment to cleave the conjugate from the resin (Figure 3A). FITC-thiol was then collected and its molecular weight was confirmed to be 436 Da by positive ion mode electrospray ionization mass spectrometry (ESI-MS). Owing to the strong gold-thiol interaction and the distance-dependent fluorescence quenching phenomenon of gold, binding of the fluorescent probe to AuNPs can be quantified by measuring the quenching of FITC emission.^[24, 25] The fluorescence emission spectrum of the FITC-thiol probe was observed at 520 nm, characteristic of FITC's emission peak. Upon mixing with citrate-stabilized AuNPs without RBC membrane coating, the fluorescence was significantly quenched, indicating the probe's close associations with gold surfaces. In contrast, when FITC-thiol was incubated with RBC-AuNPs, quenching of FITC emission was largely absent (Figure 3B). These results confirmed the presence of RBC membranes on the AuNPs and demonstrated effective shielding of gold surfaces from external thiols by the membrane coating. Compared to free FITC-thiol, the fluorescence intensity of the FITC-thiol/RBC-AuNPs mixture showed a slight decrease, which was attributable to the long-range fluorescence quenching effect of AuNPs.^[26] Notably, no further decrease in fluorescence was observed in the FITC-thiol/RBC-AuNPs mixture over a span of 72 hrs, which further confirmed the stability and the shielding effect of the RBC membrane coating.

Next, an antibody binding study was carried out to further assess the coating and sidedness of the RBC membranes on the AuNPs. Specifically, we examined the presence and orientation of CD47 on the RBC-AuNPs. CD47 is a well-documented protein marker firmly embedded in RBC membranes and is capable of inhibiting macrophage phagocytosis through interaction with the signal regulatory protein alpha (SIRP α) receptor.^[16] In the study, carboxyl-terminated polystyrene microspheres were conjugated with two distinct anti-CD47 antibodies that bind specifically to the exoplasmic and the cytoplasmic region of CD47, respectively. Following the conjugation, the microspheres were incubated with RBC-AuNPs in the presence of 1% bovine serum albumin (BSA) for 2 hrs and then washed and imaged by transmission electron microscopy (TEM). Figure 4A shows that multiple electron-dense AuNPs attached to the anti-CD47(exoplasmic) modified microspheres, confirming the presence of CD47's exoplasmic region on the RBC-AuNPs. In contrast, no binding was observed between the RBC-AuNPs and anti-CD47(cytoplasmic) modified microspheres (Figure 4B). For the uncoated AuNPs (without RBC membrane coating), no binding was observed with either the anti-CD47(exoplasmic) modified microspheres (Figure 4C) or the anti-CD47(cytoplasmic) modified microspheres (Figure 4D). These results confirm not only the RBC membrane coating on the AuNPs but also the right-side-out sidedness of the coated RBC membranes. Since it is well established that the exoplasmic and cytoplasmic sides of RBC membranes possess a charge asymmetry, with the exoplasmic side being more negatively charged,^[27] the inherent negative charge on the AuNP surfaces likely more favorably interacted with the less negative cytoplasmic side of the RBC membranes. Even though the observed antibody binding pattern provided a qualitative rather than a quantitative evaluation of the RBC membrane sidedness, a right-side-out orientation that exposed the exoplasmic side of CD47 appeared to be dominant.

After having confirmed the coating of RBC membranes on the AuNPs, we examined the functionalities of the RBC-AuNPs, particularly their anti-phagocytosis capability against macrophages. Accumulating evidence has shown that the survival of RBCs from macrophages is due to a collective contribution of multiple membrane components including CD47 [16, 28] and sialic acid [14, 29] present on the cell surfaces. Translocating natural RBC membranes onto the AuNPs is expected to bring such functionality to the resulting RBC-AuNPs. In the macrophage uptake study, RBC-AuNPs and uncoated AuNPs at a concentration of 25 $\mu\text{g/mL}$ were incubated with J774 murine macrophage cells. After 30 min of incubation, the cells were imaged to visualize particle uptake by the cells. Macrophage cells without the addition of AuNPs were prepared in parallel as a background control (Figure 5A). Incubation with RBC-AuNPs resulted in a few dark spots that corresponded to a minor particle uptake by the macrophages (Figure 5B). In contrast, incubation with uncoated AuNPs resulted in a significant number of dark spots in the intracellular and perinuclear regions of the cells, indicating that the AuNPs were actively taken up (Figure 5C). The AuNP uptake was further quantified by inductively coupled plasma mass spectrometry (ICP-MS). Under the experimental condition, RBC-AuNPs had an uptake of 3.2 ng/1000 macrophage cells, whereas an uptake of 13.5 ng/1000 macrophage cells was measured for uncoated AuNPs (Figure 5D). The reduction in AuNP uptake demonstrated the reduced susceptibility of RBC-AuNPs to macrophages, thus confirming the successful translocation of immune-evasive functionality from RBCs to RBC-AuNPs.

In summary, we report a unique and robust ‘top down’ approach to functionalize AuNPs with cellular membranes derived directly from natural RBCs, yielding AuNPs fully enclosed by continuous RBC membranes. Complete RBC membrane coating was verified through physicochemical characterizations, a thiolated fluorescent probe, and an antibody binding assay. The resulting RBC-AuNPs possess right-side-out RBC membranes and the associated membrane proteins, which bestow immunosuppressive functionalities on the AuNPs for evading macrophage uptake. In addition, the membrane coating effectively shields the particles from interacting with thiolated compounds. As applications of AuNPs in life sciences continue to expand, novel modification strategies offering advanced functionalities and features will greatly benefit the platform development. By integrating synthetic AuNPs with natural cellular membranes, the particles can be bestowed with a wide range of functionalities responsible for cells’ diverse antigenic, transport, and mechanical characteristics. The RBC-AuNPs embodies a new materials design strategy and presents an intriguing class of advanced materials for a broad range of biomedical applications.

Experimental Section

Preparation of RBC-membrane-derived vesicles

Whole blood withdrawn from male ICR mice (6–8 wk, purchased from Charles River Laboratories) was centrifuged at 800 $\times g$ for 5 min at 4°C to remove the plasma and the buffy coat. The resulting packed RBCs were washed three times with ice cold 1 $\times$ PBS prior to hemolysis via a hypotonic medium treatment. The washed RBCs were suspended in 0.25 $\times$ PBS in an ice bath for 20 min and were centrifuged at 800 $\times g$ for 5 min. The released hemoglobin was removed, whereas the pellet with light pink color was collected and washed

twice with $1\times$ PBS. The resulting RBC ghosts (devoid of cytoplasmic contents) were verified under a phase contrast microscope, which revealed an intact cellular structure with an altered cellular content. To prepare RBC-membrane-derived vesicles, the collected RBC ghosts were sonicated in a capped glass vial for 5 min using a FS30D bath sonicator (Fisher Scientific) at a frequency of 42 kHz and power of 100W, and then extruded serially through 400-nm and then 100-nm polycarbonate porous membranes with an Avanti mini extruder (Avanti Polar Lipids).

Preparation of RBC-membrane-coated gold nanoparticles (RBC-AuNPs)

Citrate-stabilized gold nanoparticles (uncoated AuNPs) with a diameter of approximately 70 nm and a concentration of 50 $\mu\text{g/mL}$ (equivalent to 0.025 nM) were purchased from nanoComposix, Inc. To fuse the RBC-membrane-derived vesicles onto the surface of AuNPs, 1 mL of AuNPs was mixed with RBC-membrane-derived vesicles prepared from 370 μL of whole blood and then extruded 7 times through a 100-nm polycarbonate porous membrane with an Avanti mini extruder. The mixture ratio was estimated based on the membrane volume of RBCs and the total membrane volume required to fully coat 1 mL of AuNPs (70 nm, 50 $\mu\text{g/mL}$). Parameters used for the estimation include density of AuNPs (19.3 g/cm^3), mean surface area of mouse RBCs ($\sim 75 \mu\text{m}^2$),^[30] membrane thickness of RBC ($\sim 8 \text{ nm}$),^[22] red blood cell concentration in mouse whole blood (~ 7 billion cells/mL).^[31] An excess of blood was used to compensate for the membrane loss during RBC ghost derivation and extrusion. The resulting RBC-AuNPs were carefully centrifuged and the excess membrane components remaining in the supernatant were removed.

Characterization of RBC-AuNPs

Particle size (diameter, nm), polydispersity, and surface charge (zeta potential, mV) were measured by dynamic light scattering (DLS) with Nano-ZS, model ZEN3600. For stability test, the RBC-AuNPs were suspended in $1\times$ PBS or 100% FBS (Hyclone) with a final particle concentration of 25 $\mu\text{g/mL}$. The size of the particles was measured in triplicate by DLS and monitored at room temperature for 72 hrs. The structure of the RBC-AuNPs was examined using a transmission electron microscope (TEM). Briefly, 1 mL of RBC-AuNPs (50 $\mu\text{g/mL}$) was carefully centrifuged to concentrate to approximately 10 μL . A drop of the concentrated RBC-AuNPs was deposited onto a glow-discharged carbon-coated copper grid. Five minutes after the sample was deposited, the grid was rinsed with 10 drops of distilled water, followed by staining with a drop of 1% uranyl acetate. The grid was subsequently dried and visualized using a FEI 200KV Sphera microscope.

Synthesis of FITC-thiol conjugate

Cysteamine 4-methoxytrityl resin (25 mg, EMD Millipore) was loaded into a solid phase reactor and pre-swelled in dimethylformamide (DMF) for 4 hrs at room temperature. NHS-Fluorescein (NHS-FITC, 12 mg, 0.025 mmol, ThermoScientific) was dissolved into dry DMF (0.3 mL) and then added to the resin together with *N,N*-diisopropylethylamine (DIEA, 20 μL , 0.11 mmol). The reaction was carried out in dark for 16 hrs and then was terminated by washing the resin with DMF followed by dichloromethane (DCM). After drying under vacuum, the functionalized resin was treated with a mixture of trifluoroacetic acid (TFA, 0.1

mL) and DCM (0.4 mL). The solvent was transferred to a glass vial and evaporated at room temperature under vacuum. The resulting FITC-thiol product was collected as a green powder and its molecular weight was confirmed by a positive ion mode electrospray ionization mass spectrometry (ESI-MS). The FITC-thiol was then incubated with RBC-AuNPs and uncoated AuNPs, respectively, and the fluorescence intensity at 520 nm was measured by an Infinite M20 multiplate reader.

Binding of RBC-AuNPs with anti-CD47 antibody modified polystyrene microspheres

To modify the surface of polystyrene microspheres with anti-CD47(exoplasmic) antibody or anti-CD47(cytoplasmic) antibody, 0.4 mg EDC and 1.2 mg sulfo-NHS were first dissolved into 200 μ L DI water and the solution was then added to 100 μ L suspension of carboxylate terminated polystyrene microspheres (diameter: 2 μ m; concentration: 4 wt%; Life Technologies Corp). After 30 min of incubation, the microspheres were washed three times with DI water to remove the excess amount of EDC and sulfo-NHS. Next, the activated microspheres were re-suspended into 100 μ L of DI water, to which 10 μ L of anti-CD47 antibody solution (0.5 mg/mL) was added. The mixture solution was allowed to react at room temperature for 2 hrs, followed by washing with DI water three times through centrifugation to remove the un-reacted antibodies. The resulting anti-CD47 antibody modified microspheres was then re-suspended into 100 μ L of DI water. The microsphere solution was first added with 1 mg of bovine serum albumin (BSA) to block unspecific binding and then mixed with 1 mL of RBC-AuNPs (50 μ g/mL). The mixture solution was incubated at room temperature for 2 hrs. After the reaction, the mixture was carefully centrifuged to remove the unbound RBC-AuNPs. The collected polystyrene microspheres were then subjected to TEM imaging. For better comparison and beauty purpose, the obtained TEM images were rotated so that the microspheres were aligned along the same direction.

Macrophage uptake study

J774 murine macrophage cells were cultured in DMEM media (Life Technologies Corp.) supplemented with 10% FBS (Sigma-Aldrich) and plated at a density of 10^5 cells/well on an 8-well chamber slide (BD Biosciences). On the day of experiment, the cells were washed and cultured in fresh culture media, followed by adding RBC-AuNPs or uncoated AuNPs with a final AuNP concentration of 25 μ g/mL. Note that at such low concentration (equivalent to 0.0125 nM), uncoated AuNPs remained stable in the culture media and thus no aggregation-induced cellular uptake would occur. Moreover, AuNPs at the tested concentration showed no cytotoxicity to the macrophage cells through an MTT assay, which is consistent with existing literatures on the cytotoxicity of AuNPs.^[32, 33] To examine macrophage uptake of RBC-AuNPs and uncoated AuNPs, the particles were incubated with the macrophage cells at 37°C for 30 min and then washed with PBS. For imaging study, the cells were then fixed with 4% glutaraldehyde at room temperature, mounted with Vectashield mounting medium, and imaged with a Zeiss 510 confocal microscope. To quantify Au uptake by the macrophage cells, the cells were lysed by adding 0.5 mL 1% Tween-80 to each well. The cell lysate from each well was then added into 1 mL aqua regia consisting of concentrated nitric acid and hydrochloric acid (Sigma-Aldrich, trace element analysis grade) in a volume ratio of 1:3. The mixture was left at room temperature for 12

hrs, followed by annealing at 80°C for 6 hrs to remove the acids. The sample was then re-suspended with 1 mL DI water and the Au content in each sample was determined by inductively coupled plasma mass spectrometry (ICP-MS).

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Figure 1. Schematic illustration shows the preparation process of gold nanoparticles (AuNPs) functionalized with RBC membranes (RBC-AuNPs). The process can be divided into two steps: deriving membrane vesicles from RBCs and fusing the vesicles onto the surfaces of AuNPs.

Figure 2. Physicochemical characterizations of RBC-AuNPs. (A) Hydrodynamic size (diameter, nm) and surface zeta potential (mV) of AuNPs before and after RBC membrane coating measured by dynamic light scattering (DLS). (B) A representative transmission electron microscopy (TEM) image showing the spherical, core-shell structure of the RBC-AuNPs under negative staining with uranyl acetate. (C) Stability of the resulting RBC-AuNPs in 100% serum and 1× PBS, respectively, by monitoring particle size (diameter, nm) over a span of 72 hrs.

Figure 3.

A fluorescence quenching assay to verify RBC membrane coating and demonstrate its shield effect. (A) Chemical synthesis of a FITC-thiol conjugate (FITC = fluorescein isothiocyanate). (B) Fluorescence spectra taken from (i) FITC-thiol alone, (ii) FITC-thiol mixed with RBC-AuNPs, and (iii) FITC-thiol mixed with uncoated AuNPs. The concentrations of FITC-thiol and AuNP are 1 nM and 0.025 nM (equivalent to 50 $\mu\text{g/mL}$), respectively.

Figure 4.

An antibody binding study to verify RBC membrane coating. TEM images of RBC-AuNPs incubated with (A) anti-CD47(exoplasmic) antibody modified polystyrene microspheres, and (B) anti-CD47(cytoplasmic) antibody modified polystyrene microspheres. In the control groups, uncoated AuNPs (without RBC membrane coating) were incubated with (C) anti-CD47(exoplasmic) antibody modified polystyrene microspheres, and (D) anti-CD47(cytoplasmic) antibody modified polystyrene microspheres. For all samples, 1 wt% bovine serum albumin (BSA) was added to inhibit non-specific adsorption of AuNPs onto the microspheres. Scale bars = 1 μm .

Figure 5. RBC membrane coating inhibits macrophage uptake. Bright field microscopic images of J774 murine macrophage cells incubated for 30 min in (A) culture medium, (B) 25 µg/mL RBC-AuNPs, and (C) 25 µg/mL uncoated AuNPs. (D) Quantitative analysis of macrophage uptake of RBC-AuNPs and uncoated AuNPs determined by inductively coupled plasma mass spectrometry (ICP-MS) measurements.