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Emerging Approaches to Functionalizing Cell Membrane-Coated Nanoparticles

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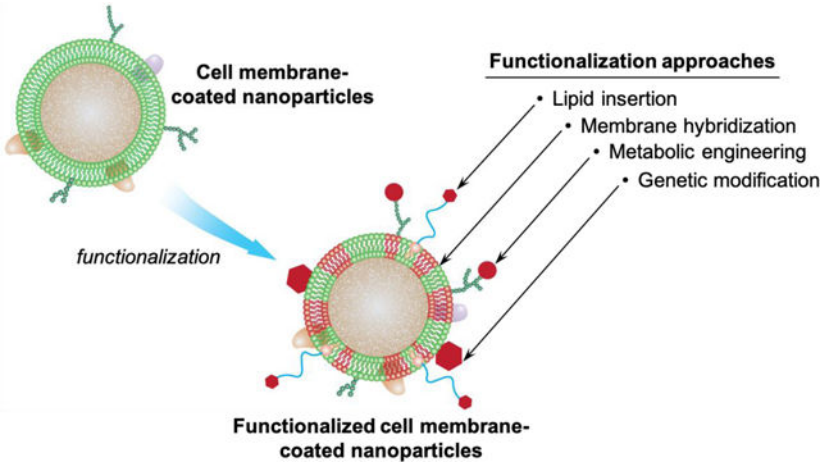
Abstract

There has been a significant interest in developing cell membrane-coated nanoparticles due to their unique abilities of biomimicry and biointerfacing. As the technology progresses, it becomes clear that the application of these nanoparticles can be drastically broadened if additional functions beyond those derived from the natural cell membranes can be integrated. Herein, we summarize the most recent advances in the functionalization of cell membrane-coated nanoparticles. In particular, we focus on the emerging methods, including (1) lipid insertion, (2) membrane hybridization, (3) metabolic engineering, and (4) genetic modification. These approaches contribute diverse functions in a non-disruptive fashion while preserving the natural function of the cell membranes. They also improve on the multi-functional and multi-tasking ability of cell membrane-coated nanoparticles, making them more adaptive to the complexity of biological systems. We hope that these approaches will serve as inspiration for more strategies and innovations to advance cell membrane coating technology.

Graphical Abstract



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Keywords

Nanotechnology; nanomedicine; nanoparticle; surface functionalization; cell membrane

Introduction

Interest in developing therapeutic nanoparticles has grown for decades, motivated primarily by their potential applications to improve disease diagnosis, treatment, and prevention.[1] Among various platforms, cell membrane-coated nanoparticles, made by wrapping natural cell membranes onto synthetic nanoparticulate cores, have attracted much attention.[2] This platform stands out because of its ability to replicate the highly complex cellular functionalities to create new therapeutic modalities.[3] For example, by inheriting 'markers of self' from the source cells, some cell membrane-coated nanoparticles effectively evade immune clearance, becoming superior long-circulating drug carriers.[4, 5] Some inherit exquisite affinity ligands native to the parent cells, becoming capable of actively targeting the disease sites.[6, 7] Some act as cell decoys to intercept harmful molecules or pathogens and protect source cells without the prior knowledge of the threat. This mechanism has allowed for a function-driven and broad-spectrum detoxification strategy.[8] By mimicking parent cells, some cell membrane-coated nanoparticles offer faithful and more relevant antigen presentation.[6, 9, 10] Some can also detain bacterial toxins to restrict their harm while preserving their structural integrity.[11, 12] These unique abilities allow them to work as vaccines that elicit highly effective protective immunity.

Since their initial development, cell membrane-coated nanoparticles are increasingly applied to complex biological systems. This leads to an increased demand for multi-functionality and multitasking. In some scenarios, providing additional functions or functional ligands seems beneficial to boost the performance of these nanoparticles. For example, while the cell membrane coating offers impressive stealth and immune evasion, additional target-selectivity may further limit off-target side effects and enhance treatment efficacy.[13, 14] While these nanoparticles can faithfully present antigenic information for immune uptake, additional control over the amplitude of the immune activation would be desirable to modulate immunity.[15, 16] Furthermore, other functionalities such as those responsive to environmental stimuli, if available, would provide cell membrane-coated nanoparticles with a more dynamic and intelligent biointerfacing capability.[17, 18] Clearly, functionalities beyond the natural properties of cell membranes, if added, would significantly expand the application of this novel class of nanoparticles.

To introduce additional functionalities, researchers have developed conjugation methods that employ amine-, carboxyl-, biotin-, or sulfhydryl-based reactions.[19, 20] These methods are convenient to decorate the cell membrane with functional ligands. However, they lack control over the position and density of the linked ligands. Random chemical reactions tend to cause cell membrane damage such as membrane protein aggregation or undesirable exposure of phosphatidylserine to the outer leaflet of the membrane bilayers, which compromises immune integrity.[21, 22] Sequential conjugations by first anchoring linkers onto the cell membrane followed next by ligand conjugation to the linkers have been developed.[23]

Although the method can minimize membrane damages, it may also limit ligand choices and density in the conjugation.

Challenges faced by traditional ligand conjugation have also motivated a few non-disruptive and straightforward strategies well suited for functionalizing cell membrane-coated nanoparticles (Figure 1). Specifically, these methods include (1) the lipid insertion method that incorporates functional ligands by first synthesizing a ligand-linker-lipid conjugate and then inserting the lipid tether into the membrane bilayers, (2) the membrane hybridization method that fuses membranes of different cell types to combine complementary ligands for functionalization, (3) the metabolic engineering method that allows the ligand to participate in natural oligosaccharide or lipid synthesis pathways for expression onto the cell membrane, and (4) the genetic modification method that expresses protein ligands onto the cell surface through gene editing. In this article, we discuss the principles of each method and summarize their recent development with an emphasis on how the introduced and native functionalities cooperate for a better therapeutic outcome. We conclude the article with a discussion on potential future development. As time progresses, these methods will undoubtedly inspire new functionalization approaches and broader applications of cell membrane-coated nanoparticles.

Lipid Insertion

Lipid insertion refers to a method that incorporates functional ligands onto natural cell membranes through a lipid anchor. Functional moieties can be conjugated to the anchor before mixing with the membrane.[24] By exploiting the fluidity of bilayered lipid membranes, the insertion relies on physical rather than chemical interactions for membrane anchoring. Sonication or extrusion commonly used for membrane coating can facilitate the lipid insertion. In addition, ligand density can be precisely tailored by controlling its initial input, a property beneficial to formulation optimization. These advantages altogether make lipid insertion attractive for functionalizing cell membrane-coated nanoparticles.

Lipid insertion has been used to anchor a variety of affinity ligands onto cell membrane-coated nanoparticles to achieve targeting ability. For example, folate and aptamers were conjugated with 1,2-disteroyl-sn-glycero-3-phosphoethanolamine-*N*-[amino(polyethylene glycol)] (DSPE-PEG) and inserted into red blood cell (RBC) membranes for cancer targeting. The resulting membrane-coated nanoparticles bound to cancer cells in a ligand-specific manner, confirming the successful transfer of biological function onto the nanoparticles.[25] Using lipid insertion to anchor small molecule ligands for functionalization has become popular due to the structural simplicity of the ligands and their easy conjugation to the lipid.[26] Following this initial development, other small molecules have been used for functionalization, including mannose and binding peptides.[27–37] In addition to the RBC membranes, cancer cell membranes have also been demonstrated for ligand insertion.[38, 39] Targeted diseases have subsequently expanded to include more types of cancer, such as melanoma and glioblastoma, and other diseases, such as stroke. Lipid insertion further allowed nanoparticles to facilitate a two-step ‘pre-targeting’ strategy aimed towards enriching imaging agents at the tumor site. [40] In this work, RBC membranes were inserted with two ligands: folate and an azide. The membrane-coated nanoparticles were

first directed to the tumor site by the folate, where they served as a homing agent to attract azide-reactive dibenzocyclooctyl (DBCO)-modified imaging agents.

In addition to small-molecule ligands, lipid insertion has been applied to anchor antibodies onto the surface of cell membrane-coated nanoparticles for targeting. In this case, lipid molecules are first linked with functional groups reactive to antibodies such as aldehyde, amine, thiol, and carboxyl groups, and used subsequently for insertion.[19, 20] With this approach, antibodies were inserted to target human epidermal growth factor receptor 2 (HER2), epithelial growth factor receptor (EGFR), and epithelial cell adhesion molecule (EpCAM) on cancer cells.[41–44] Notably, when compared with small-molecule ligands, antibodies are bulkier. Their geometric orientation is more challenging to control because available functional groups can randomly distribute over the protein surface. In this regard, modification of the reactivity across the antibody surfaces for site-selective conjugation can improve the control of antibody orientation.[45] Meanwhile, antibody fragments could serve as an attractive alternative to replace full-size antibodies in lipid insertion for their smaller sizes and better control over the conjugation sites.[46]

For lipid insertion, most applications have used 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (PEG-DSPE) as the lipid anchor, with a PEG spacer added to preserve the freedom of the ligand for bioactivity.[23, 47] Streptavidin is often used as an additional linker between the lipid and the ligand. For the multivalency of streptavidin, each lipid could anchor up to four biotinylated ligands.[48] With streptavidin-biotin chemistry, the lipid can be biotinylated for insertion, followed by linking with the streptavidin-conjugated ligands.[43] On the other hand, the lipid can be linked with streptavidin first for insertion, followed by conjugation with biotinylated ligands.[49] With a molecular weight of 60 kDa, streptavidin is relatively large. It also has a neutral charge at physiological pH. Therefore, when used as a linker, streptavidin blocks some interactions between the positively charged ligand and the negatively charged cell membranes that otherwise may restrict the freedom of the ligand and hinder its bioactivity. For example, ^{D}CDX peptide derived from candoxin targets the nicotinic acetylcholine receptors (nAChR) on the brain endothelial cells.[50] However, with a strong positive charge, the peptide interacts with the cell membrane, making it unsuitable for direct lipid insertion. Using lipid with a streptavidin linker blocked such unwanted interactions and successfully targeted the nanoparticles to the brain (Figure 2).

Besides serving as the anchor for the ligands, the lipid itself can also carry functions that, after insertion, alter cell membrane properties in response to environmental stimuli such as light, oxygen level, and pH for desirable purposes. For example, a lipid molecule, 1,1’-Dioctadecyl-3,3,3’,3’-Tetramethylindotricarbocyanine iodide (DiR), was inserted into RBC membranes to convert near-infrared (NIR) into heat and induce local hyperthermia (Figure 3).[51] The nanoparticle core was prepared with a thermo-sensitive lipid 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) with a transition temperature around 41.5°C. Without NIR light irradiation, DiR did not generate heat, and the nanoparticle core remained intact. However, under NIR light, DiR produced thermal energy to trigger the phase transition of DPPC, which destroyed the nanoparticle core for drug release. In another example, DSPE-PEG was conjugated with a TGF β-neutralizing antibody through a hypoxia-sensitive

azobenzene linker. In the normoxia environment, the nanoparticle retained the antibodies on its surface. However, in the hypoxic environment of the bone marrow, the azobenzene linker was cleaved, releasing the TGF β-neutralizing antibodies to block signaling between leukemia cells and adjacent niche cells.[39] As another example, liposomes incorporating a pH-sensitive lipid, DSPE-polyethyloxazoline (PEOz), was co-extruded with platelet membrane to form DSPE-PEOz-inserted "platesomes".[52] The PEOz moiety can be rapidly protonated at endo-lysosomal pH, generating electrostatic repulsion to de-stabilize the membrane structure and release the therapeutic payload.

Studies of using lipid insertion to functionalize cell membrane-coated nanoparticles are summarized in Table 1. Overall, this method is efficient and straightforward, offering tremendous versatility to functionalize cell membrane-coated nanoparticles. The lipids not only serve as anchors for the ligands but can also carry unique functionalities, especially those that are environment-responsive. To modulate ligand density, monovalent or multivalent linkers are available. The method has been proven successful for anchoring different ligands with varying physicochemical properties and biological functions. As the method becomes increasingly popular, fundamental understanding on ligand-membrane interactions has also improved the rationale selection of ligands for insertion toward *in vivo* applications. The lipid insertion method is expected to bring in tremendous opportunities for the development and use of functionalized cell membrane-coated nanoparticles.

Membrane Hybridization

A variety of cell membranes have been successfully utilized for nanoparticle coating.[2] The success has also motivated the recent development of mixing multiple cell membranes to develop 'hybrid membranes' aimed at boosting the functional characteristics of coated nanoparticles.[53] One way of making such hybrid membranes is to first derive the membrane from individual cell types and then fuse them through mechanical forces such as stirring, extrusion, or sonication.[53–55] Alternatively, hybrid membranes can be made by first fusing different live cells, followed by deriving the membrane from the cell hybrids.[56, 57] Nanoparticles coated with hybrid membranes inherit the virtues of each parent cell type and harness the complementary functionalities (Figure 4).[53] In various applications, these nanoparticles have shown better performances when compared with their counterparts coated with the individual membrane.[54–58]

Membrane hybridization has been used to introduce affinity ligands unique to one cell type to another, therefore adding targeting ability to the hybrid membrane-coated nanoparticles. In this regard, the platelet membrane is a popular choice for platelet receptors such as P-selectin, glycoprotein IIb/IIIa, and C-type lectin-like receptor 2 (CLEC-2) for specific tumor targeting.[59–61] For example, platelet membranes were hybridized with RBC membranes and coated onto synthetic liposomes for the co-delivery of a sonosensitizer and a cytotoxic compound for anti-cancer sonodynamic therapy.[62] In tumor-bearing mice, the hybrid membrane endowed a tumor-targeting capability, leading to a higher level of drug accumulation at the tumor site. Platelet membranes were also hybridized with neutrophil membranes, and their hybrid membranes were coated onto gold nanocages for the delivery of cytotoxic drugs and photosensitizers.[63] In this case, the neutrophil membrane contributed

additional targeting ability by recognizing multiple adhesion molecules on circulating tumor cells (CTCs) such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1).[63, 64] The nanocages coated with hybrid membranes showed greater cellular uptake, deeper tumor penetration, and higher cytotoxicity when compared to non-coated or single membrane-coated gold nanocages.[63]

Membrane hybridization has also been used to boost immune evasion of the nanoparticles by bringing in another membrane with a stronger stealth ability. For example, cancer cell membrane-coated nanoparticles (CCNPs) have become a popular delivery platform for tumor binding.[6] However, CCNPs made from cancer cell membranes alone do not seem stealthy enough to evade immune surveillance, mostly attributed to their possession of tumor-specific antigens on the membrane surface.[65] As a result, their efficacy is limited by the rapid phagocytic clearance in the circulation. To address this shortcoming, researchers hybridized cancer cell membranes with RBC membranes that brought in ‘markers of self’ such as CD47 to enhance the stealth capability of the hybrid membrane.[66] Nanoparticles coated with such cancer cell-RBC hybrid membranes showed a prolonged circulation half-life and a higher level of accumulation at the tumor site. Besides RBCs, platelets and leukocytes are also known for their prominent immune evasion. Recently, their membranes were hybridized with cancer stem cell membranes. The resulting hybrid membrane-coated nanoparticles showed longer circulation times compared to their CCNP counterparts.[65, 67]

Membrane hybridization has also been used to bring in ‘homologous’ characteristics aimed at reducing undesirable cell-binding interactions. For example, CCNP were used to capture and isolate circulating tumor cells (CTCs) for their unique homotypic binding.[68] However, the competitive binding between CCNP and white blood cells (WBCs) limited the detection sensitivity and capture efficiency. To overcome this challenge, researchers hybridized cancer cell membranes with membranes of WBCs.[68] By being ‘homologous’ to WBCs, the hybrid-membrane coated nanoparticles had significantly reduced interference from WBCs. The capture efficiency and detection sensitivity toward CTCs were improved considerably. Similar to cancer cells, platelets also bind with CTCs specifically.[59–61] Therefore, platelet membrane-coated nanoparticles (PNPs) were also applied for CTC capture and isolation.[58] In this approach, leukocytes compete with CTCs to bind with PNPs, reducing the isolation efficiency. This challenge was addressed by hybridizing platelet membranes with leukocyte membranes. By possessing features homologous to the leucocytes, hybrid membrane-coated nanoparticles showed less undesirable binding, and the CTC isolation efficiency was improved.

Membrane hybridization has also been used to incorporate immune-stimulatory properties for improving the outcome of immunotherapy. In particular, CCNPs with an array of tumor antigens present on their surfaces have been explored as anti-cancer vaccines.[6, 10] However, the anti-tumor response induced by CCNPs is often hampered by downregulated antigen expression and tumor heterogeneity. Hybridization of the cancer cell membrane with a secondary membrane has been used to enhance the immunogenicity. [56, 57] For this purpose, attenuated *Salmonella* outer membrane vesicles (OMVs) were hybridized with cancer cell membranes and coated onto nanoparticles, resulting in a tumor-specific antigenic nanoplatform with self-adjuvanting activities.[69] In mouse melanoma models, the hybrid

membrane-coated nanoparticles induced anti-cancer immunity boosted by both dendritic cells (DCs) and cytotoxic T cells. Somatic hybrids of DCs and cancer cells were also made to produce hybrid membranes (Figure 5).[56] Nanoparticles coated with such hybrid membranes acquired the antigen-presenting ability of DCs and therefore enhanced both direct and DC-mediated T cell activation for better anti-cancer immunity. This platform also combined cancer immunotherapy with photodynamic therapy and showed promising efficacy.[57]

Studies of using the hybrid membrane to functionalize nanoparticles are summarized in Table 2. This method provides nanoparticles with functionalities otherwise exclusive to individual cell membranes. A variety of membrane combinations have been studied, creating synergies by combining an array of functions such as long circulation with active targeting and antigen presentation with immune stimulation.[54, 56, 57, 65, 67] With abundant cell membranes to choose from, this method provides great flexibility in designing tailored and personalized nanomedicines. Future development is increasingly focused on understanding the membrane composition-efficacy relationship while improving the precision and reproducibility of the membrane hybridization process.[2, 70] With continuous development, hybrid membrane-coated nanoparticles are expected to make a more significant impact on future clinical applications.

Metabolic Engineering

Metabolic engineering aims to control cellular properties through manipulating cells' natural biosynthetic pathways. For cell membrane modification, metabolic substrates are first conjugated with functional moieties and then incubated with cells for uptake and metabolism.[71–73] These non-natural conjugates hijack natural biosynthesis pathways, participate in the relevant cellular metabolic processes, and subsequently anchor onto the cell surfaces.[74–76] Based on this principle, glycoengineering relies on oligosaccharide and glycoconjugate productions, including fucose salvage, sialic acid, and *N*-acetylgalactosamine (GalNAc) salvage pathways, to modify cell membranes (Table 3).[77–79] Monosaccharides substrates such as *N*-acetylmannosamine (ManNAc), *N*-acetylneuraminic acid (Neu5Ac), GalNAc, and fucose are commonly used to form conjugates with functional moieties for metabolism.[80–83] Meanwhile, lipid engineering exploits natural lipid synthesis such as the cytidine 5′-diphosphocholine (CDP-choline) pathway for membrane modification, where moieties are commonly conjugated with choline analogs for metabolism.[84–86] Through metabolic engineering, various functional moieties, especially bioorthogonal linkers, have been installed onto the membrane surface for desirable functionalities.[87–89]

Recently, the glycoengineering was used to functionalize cell membrane-coated nanoparticles for tumor targeting *in vivo*. In this work, the tetraacetylated N-azidoacetylgalactosamine (Ac_4GalNAz) was treated with T cells to introduce azide groups on cell membranes through the natural GalNAc salvage pathway (Figure 6).[90] Following this modification, the N_3-labeled T cell membranes were derived and coated onto PLGA cores pre-loaded with a photosensitizer (denoted 'N_3-TINPs'). Meanwhile, bicyclo [6.1.0] nonyne (BCN)-modified mannose substrate (Ac_4ManN-BCN) was injected into the tumor region.

Through the sialic acid pathway, the substrates were taken up by the tumor cells, and the BCN group was expressed onto the tumor surfaces. Following the injection of the N_3-TINPs, the selective click reaction between BCN and N_3 groups facilitated specific homing of the N_3-TINPs to the tumor region. Such tumor-specific homing is further facilitated through the immune recognition of CD3 on the T cells membrane of N_3-TINPs by the tumor cells. Equipped with such targeting mechanisms, N_3-TINPs accumulated at a higher level in the tumor region after their intravenous administration when compared with nanoparticles coated with unmodified T cell membranes (denoted 'TINP'). When tested for *in vivo* photothermal therapeutic efficacy, N_3-TINPs also showed more significant tumor inhibition with negligible adverse effects compared with TINP.

Phospholipid engineering was also utilized to introduce bioorthogonal linkers on the membrane-coated nanoparticles, which allowed for the further conjugation of immune stimulator ligands. In this study, an azide-choline substrate was applied to add N_3 groups on the leukocyte membrane through the CDP-choline biosynthesis pathway (Figure 7A).[91] Following the expression, N_3-labeled membrane was then coated onto magnetic nanoclusters (MNCs). Through the click reaction, N_3-tagged MNCs were further conjugated with major histocompatibility complex class-I (pMHC-I) and co-stimulatory ligand anti-CD28. With the presence of both ligands, the nanoclusters acted as artificial antigen-presenting cells (aAPCs) and induced a significant increase of $CD8^+$ T cell proliferation when compared to free anti-CD28. The T cells activated by nanoclusters were intravenously injected into the EG7 tumor-bearing mice. These mice showed slower tumor growth and a better survival rate when compared with the control group injected with T cells activated by free antibodies. The versatility of phospholipid engineering for functionalizing membrane-coated nanoparticles was demonstrated in another study, where the same phospholipid pathway was used to express N_3 groups on macrophage membranes (Figure 7B).[92] Following the modification, the membrane was coated onto MNC-siRNA nanocomplex. Through click chemistry, the nanocomplex was further conjugated with an RGD peptide that targets integrin $\alpha_v\beta_3$ over-expressed on the tumor. When intravenously injected, the targeted nanocomplex showed a 2.7-fold increase of tumor accumulation as well as a significant inhibition of tumor growth compared to nanoparticles coated with unmodified membranes.

Overall, recent development has demonstrated metabolic engineering as an agile and versatile approach to harnessing natural biosynthesis pathways for ligand expression onto cell membrane-coated nanoparticles. Functionalization applications with metabolic engineering are expected to grow as novel ligands compatible with biosynthesis are continually discovered, and methods for enforced ligand expression are continually developed.[93] Meanwhile, different ligands can be simultaneously installed by using substrates of non-overlapping pathways, potentially increasing the spectrum and capacity of drug targeting or detoxification.[94] In addition to mammalian cells, metabolic engineering can also be applied to modify bacterial membranes. For example, modifying non-pathogenic bacteria to express surface glycans of pathogenic strains becomes attractive to modulate membrane self-adjuvanticity.[95] Towards future development, the progress made in metabolic engineering will bring in new tools and strategies to functionalize cell membrane-coated nanoparticles for broader applications.

Genetic Modification

Genetic modification is a powerful method to acquire new functions by altering the protein expression on the cell surfaces. Through selective gene editing, genetically modified membranes (namely 'GM membranes') can be made and coated onto nanoparticles for functionalization. Genetic modification can use robust cell lines to express unique antigens native to sensitive cells, which may lower the cost for large-scale manufacturing.[96] For gene modification, DNA or mRNA materials need to access the cytosol. Such intracellular delivery can be accomplished by using a variety of methods (Table 4). For example, viral vehicles, including those based on adenovirus, lentivirus, and adeno-associated virus, offer superior efficiency of the transfection.[97–101] For better safety, synthetic materials such as cationic lipids or polymers have also been developed for intracellular delivery.[102–105] Meanwhile, physical methods, including electroporation, gene gun, laser-irradiation, and microinjection, are popular.[106–110] Recently, these methods were combined with CRISPR/Cas9 technology, resulting in faster, cheaper, more accurate, and more efficient gene editing capability.[111, 112]

The genetic modification method can express highly specific affinity ligands to provide cell membrane-coated nanoparticles with targeting capability. For example, hepatitis B virus (HBV) preS1 ligand was expressed onto HepG2 cells, after which the membrane was derived and coated onto oncolytic adenoviruses (OAs, Figure 8A).[113] Such membrane coating decreased the immunogenicity of OA without compromising their infectivity for tumor inhibition. It also allowed the GM-coated viruses to target tumors with active overexpression of preS1 receptor (NTCP). In the study, intravenous administration of GM-coated OAs resulted in a higher tumor accumulation and anti-cancer efficacy compared to uncoated viruses (Figure 8B and C). To further demonstrate the adaptability of using GM for viral targeting, a small peptide Asn-Gly-Arg (NGR), was expressed onto RBC membranes through in- body CRISPR technology. The peptide targets a specific isoform of aminopeptidase N (APN), a membrane metalloproteinase on a variety of cancer cells. Following the genetic modification, the GM-membranes were coated onto OAs. This time, GM membrane-coated OAs showed significant increases in tumor accumulation and tumor growth inhibition in APN receptor-expressing tumors, including PC13, U87, and HepG2 tumors, in comparison with uncoated OAs (Figure 8D).

The genetic modification method can also express surface moieties aimed at prolonging nanoparticle *in vivo* circulation times. Traditionally, surface functionalization with PEG is a popular approach. However, concerns about PEG functionalization, such as the high cost of manufacturing and the secondary immunogenicity, are growing. As a potential alternative, peptide-based polymers such as natural *L*-amino acid chains containing Pro, Ala, and Ser (PAS) have shown stealth properties comparable to that of PEG.[114] More compelling, these polymers can be produced by genetically encoded biosynthesis. Recently, PAS chains were expressed onto HEK293 cells with a plasmid encoding a fusion protein, PAS repeats, and a C-terminal transmembrane anchoring domain (Figure 9A).[115] The GM membranes expressing PAS were derived and coated onto PLGA cores. The resulting nanoparticles (PASylated nanoghosts) showed a significant reduction in BSA adsorption and macrophage uptake when compared to those coated with wildtype HEK293 membranes. When tested *in*

vivo, nanoparticles coated with GM membranes showed a three-fold increase in circulation half-life when compared to those coated with wild type membranes (Figure 9B). The percentage distribution of the sample groups in different organs at 48 h post-injection is shown in Figure 9C. All sample groups showed similar biodistribution profiles, with the majority of accumulation being observed in the liver.

Overall, cell membrane coating provides a technology platform that harnesses the merits in nanotechnology and genetic engineering. This advance is especially promising to make nanoparticles with 'universal' membranes, where membranes of allogeneic cells can be used for coating after selectively knocking out antigen-presenting proteins such as MHC I and II.[116, 117] In addition, recent development in expressing viral antigens onto mammalian membranes and bacterial engineering for selective antigen expression on their outer membranes can be potentially used to make GM membrane-coated nanoparticles for better modulating anti-viral or anti-bacterial immunity.[118–120] Overall, the combination of genetic engineering with cell membrane coating technology is expected to generate exciting innovations for future therapeutics.

Conclusions

As cell membrane-coated nanoparticles are increasingly developed for various biomedical applications, approaches to further functionalizing these biomimetic nanoparticles are emerging. In this article, we highlighted four unique methods, including lipid insertion, membrane hybridization, metabolic engineering, and genetic modification. Despite their different underlying principles, these methods all feature non-disruptive functionalization procedures compatible with existing membrane derivation and coating processes. We summarized the applications of each method with a specific emphasis on how the approach confers cell membrane-coated nanoparticles with more functions beyond those from the native cell membranes. Overall, these methods improve on the multi-functional and multi-tasking ability of cell membrane-coated nanoparticles, making them more adaptive to the complex biological systems.

As the nanoparticle functionalization strategies emerge, cell membrane coating technology has also made significant progress. For example, cell membrane-coated nanoparticles are increasingly combined with other materials such as hydrogels for local applications.[121–123] Methods aimed at modifying the cores rather than the membranes have also been applied to enhance overall nanoparticle functionality.[124] Meanwhile, cell membranes are increasingly used to coat self-propelled and autonomous nanomotors, opening a variety of *in vivo* applications.[125, 126] Cell membranes have also been coated onto biomaterials with higher dimensions such as nanofibers and planary devices.[127, 128] We believe that the strategies for functionalizing cell membrane-coated nanoparticles can also be applied to these new directions. For example, RBC-platelet hybrid membranes were recently coated onto nanomotors for concurrent removal and neutralization of pathogenic bacteria and toxins.[129] Mechanistic studies have revealed that nanoparticle surface functionalization plays dynamic roles in altering the patterns and pathways of nanoparticle interactions with cells. Therefore, selecting an appropriate functionalization method may help to target specific intracellular pathways for better therapeutic interventions with reduced side effects.[130, 131] Overall,

we expect these emerging surface functionalization approaches discussed above to play significant roles as researchers continue to refine and expand cell membrane coating technology towards broader applications.

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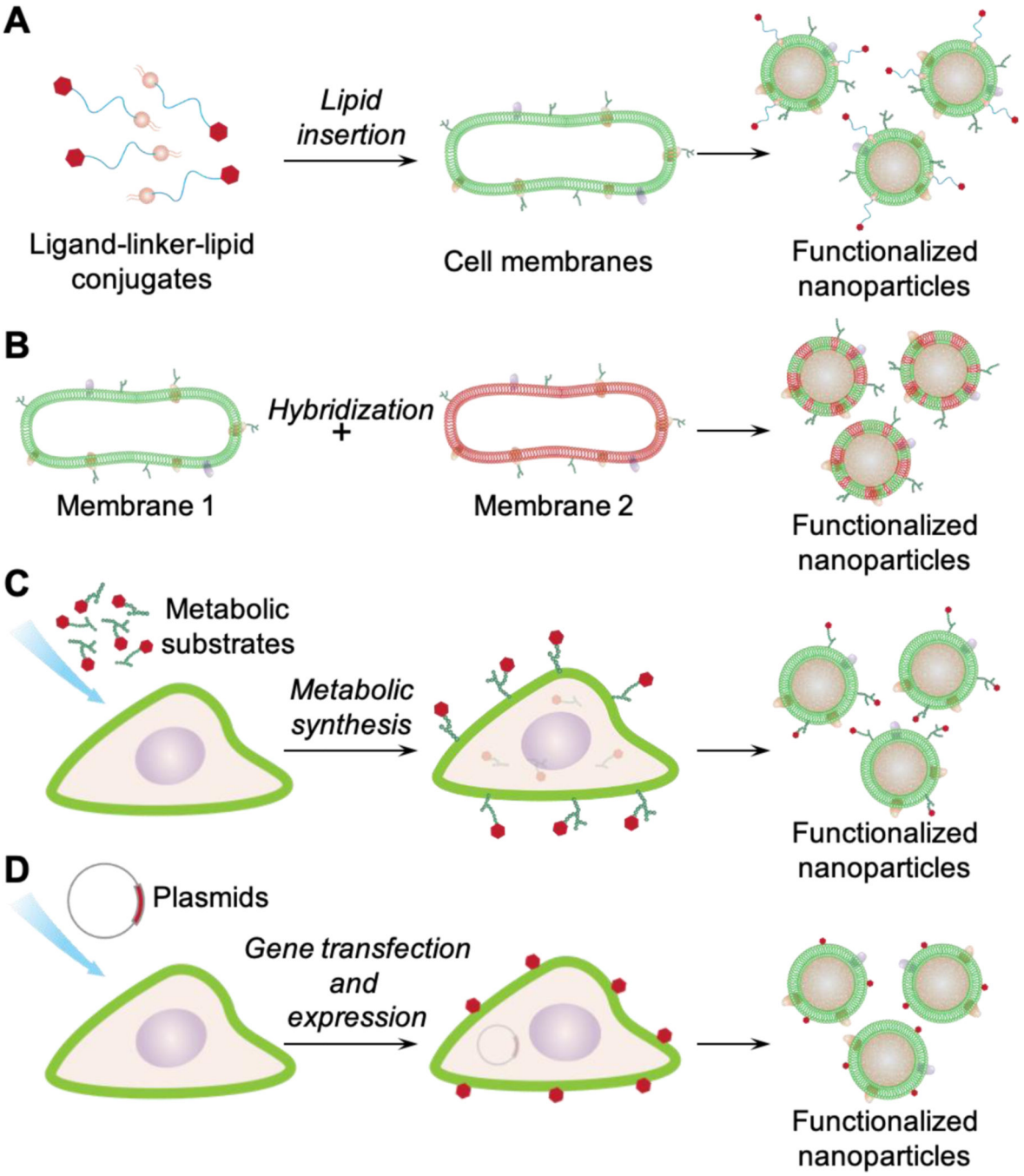


Figure 1.
Schematic showing different methods for functionalizing cell membrane-coated nanoparticles. (A) lipid insertion, (B) membrane hybridization, (C) metabolic engineering, and (D) genetic modification.

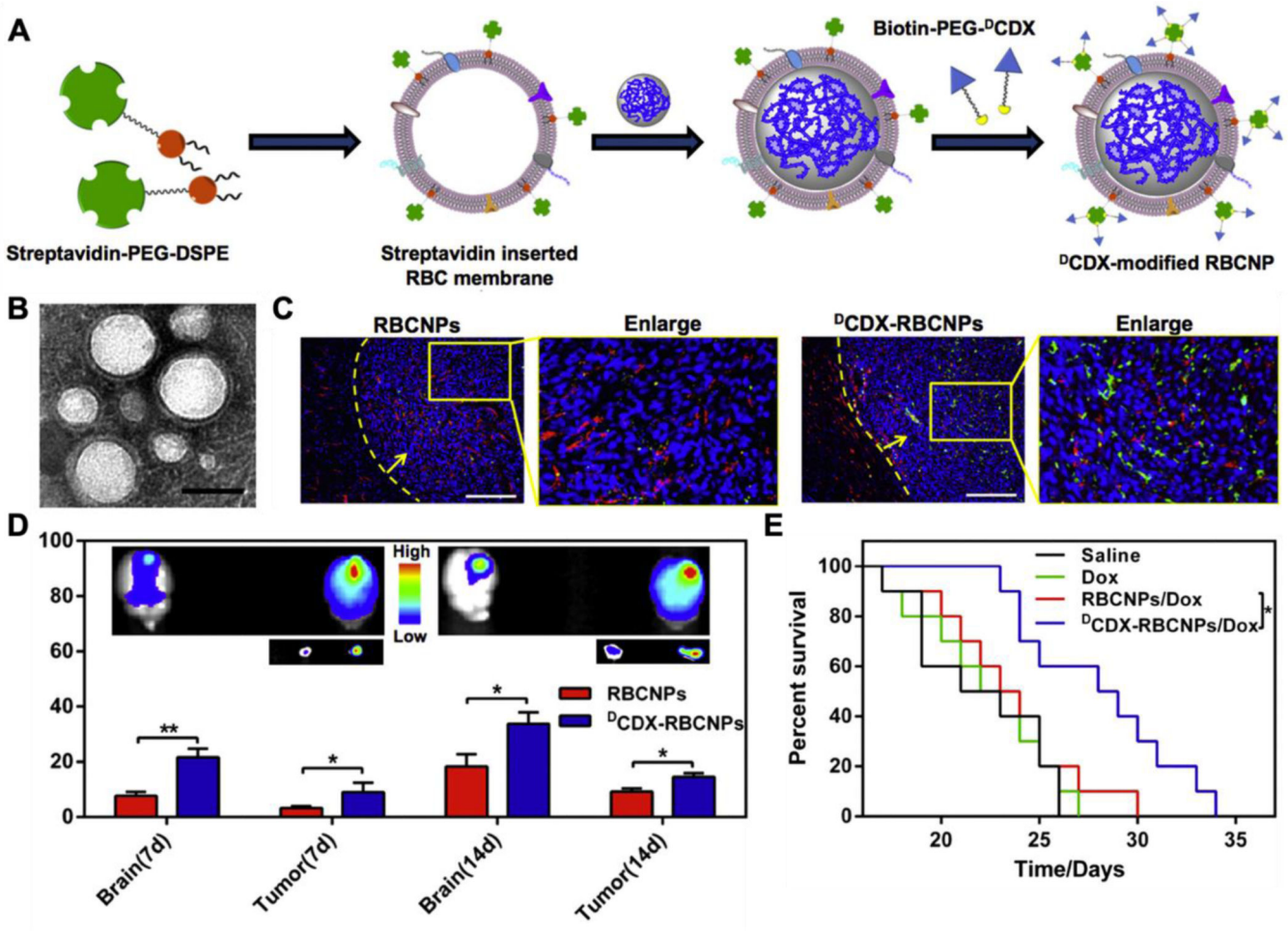


Figure 2.

(A) Schematic of the preparation of nanoparticles coated with ^{D}CDX-modified RBC membranes (^{D}CDX-RBCNPs). Streptavidin-PEG-DSPE is synthesized and then inserted into RBC membranes. After coating polymeric cores, biotin-PEG-^{D}CDX binds to the streptavidin on the surface of the resulting RBCNPs to form ^{D}CDX-modified RBCNPs. (B) Transmission electron microscope image of ^{D}CDX-RBCNPs. (C) The distribution of nanoparticles in the brain of tumor-bearing mice 14 days post-implantation. Nuclei were stained with DAPI (blue), blood vessels were labeled with anti-CD31 (red), while green represents the DiI-loaded nanoparticles. The yellow dotted lines represent the margins of the glioma and the yellow arrows point to the glioma (scale bars, 200 μm). (D) *Ex vivo* images and average radiant efficacy of brains and tumors in tumor-bearing mice (7 or 14 days after implantation). Bars represent means with SD, n = 3, *p < 0.05, **p < 0.005. (E) Kaplan-Meier survival curves of nude mice bearing intracranial U87 glioma. Mice (n = 10) were injected at 7, 9, 11, 13 and 15 days after glioma implantation with saline, free Dox, Dox-loaded RBC-NPs (RBCNPs/Dox), and Dox-loaded ^{D}CDX-RBCNPs (^{D}CDX-RBCNPs/Dox). Reproduced with permission from ref 50. Copyright 2017 Elsevier.

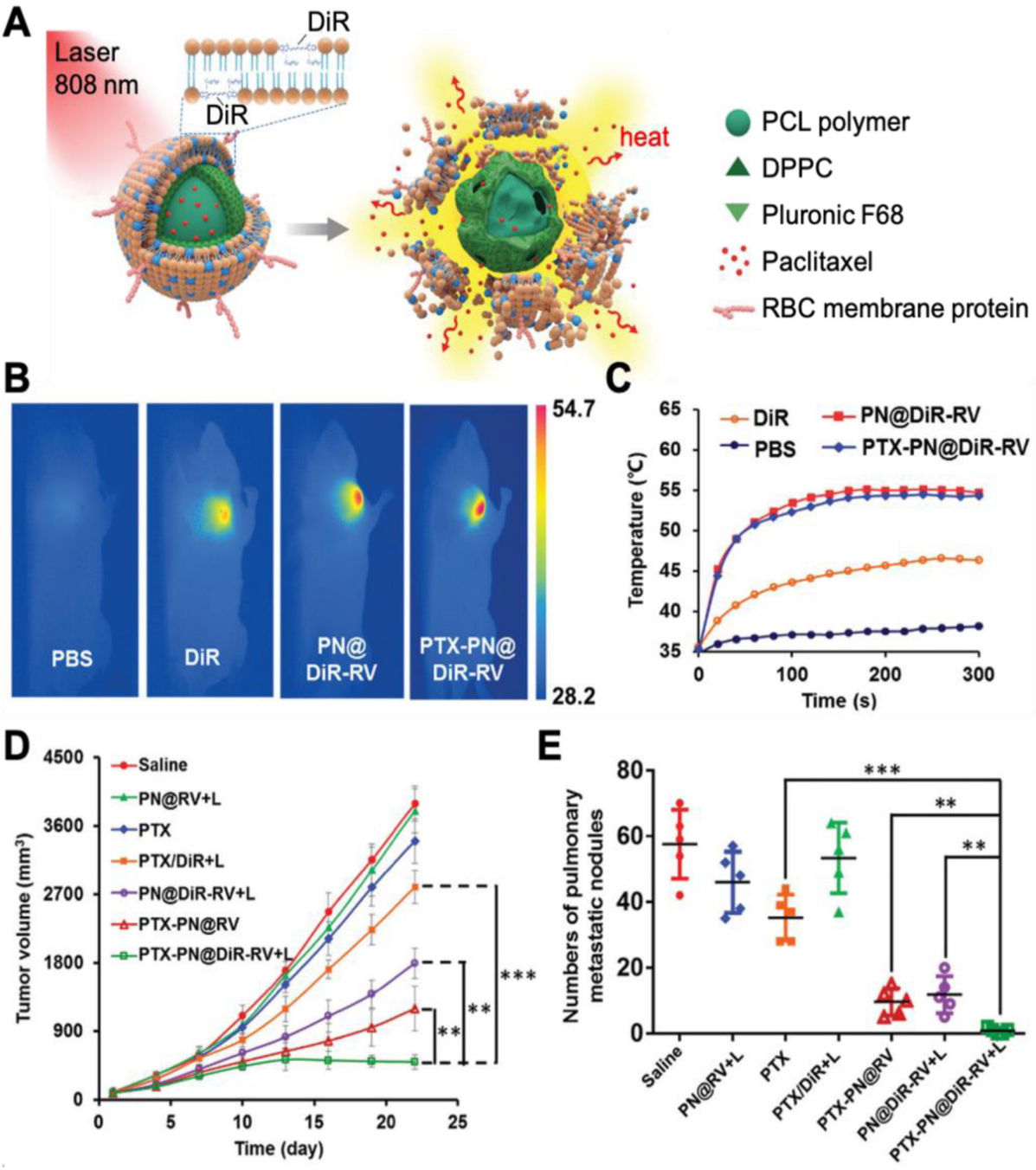


Figure 3.

(A) The near infrared light (NIR)-driven drug release of the red blood cell (RBC) membrane-coated nanoparticles (PTX-PN@DiR-RV). DiR dye was embedded in the RBC membrane (DiR-RV), and the thermosensitive lipid DPPC was added to the polymeric cores (PN). Under the 808 nm laser irradiation (+L), DiR provided strong thermal energy and then triggered the phase transition of DPPC, leading to the destruction of the cores and the release of paclitaxel (PTX). (B) The infrared thermographic images of mice after 4 h i.v. injection with PBS, free DiR, PN@DiR-RV, and PTX-PN@DiR-RV, respectively.

(C) The temperature elevation profile of each group in (B). (D-E) *In vivo* antitumor and anti-metastasis efficacy by the synergetic chemo-photothermal therapy of PTX-PN@DiR-RV. (D) Tumor growth of mice after intravenous injection of different formulations. (E) Quantitative analysis of the lung metastatic nodules for each group. Data were presented as mean ± SD (n = 6), ** $P < 0.01$, *** $P < 0.005$. Reproduced with permission from ref 51. Copyright 2016 John Wiley and Sons.

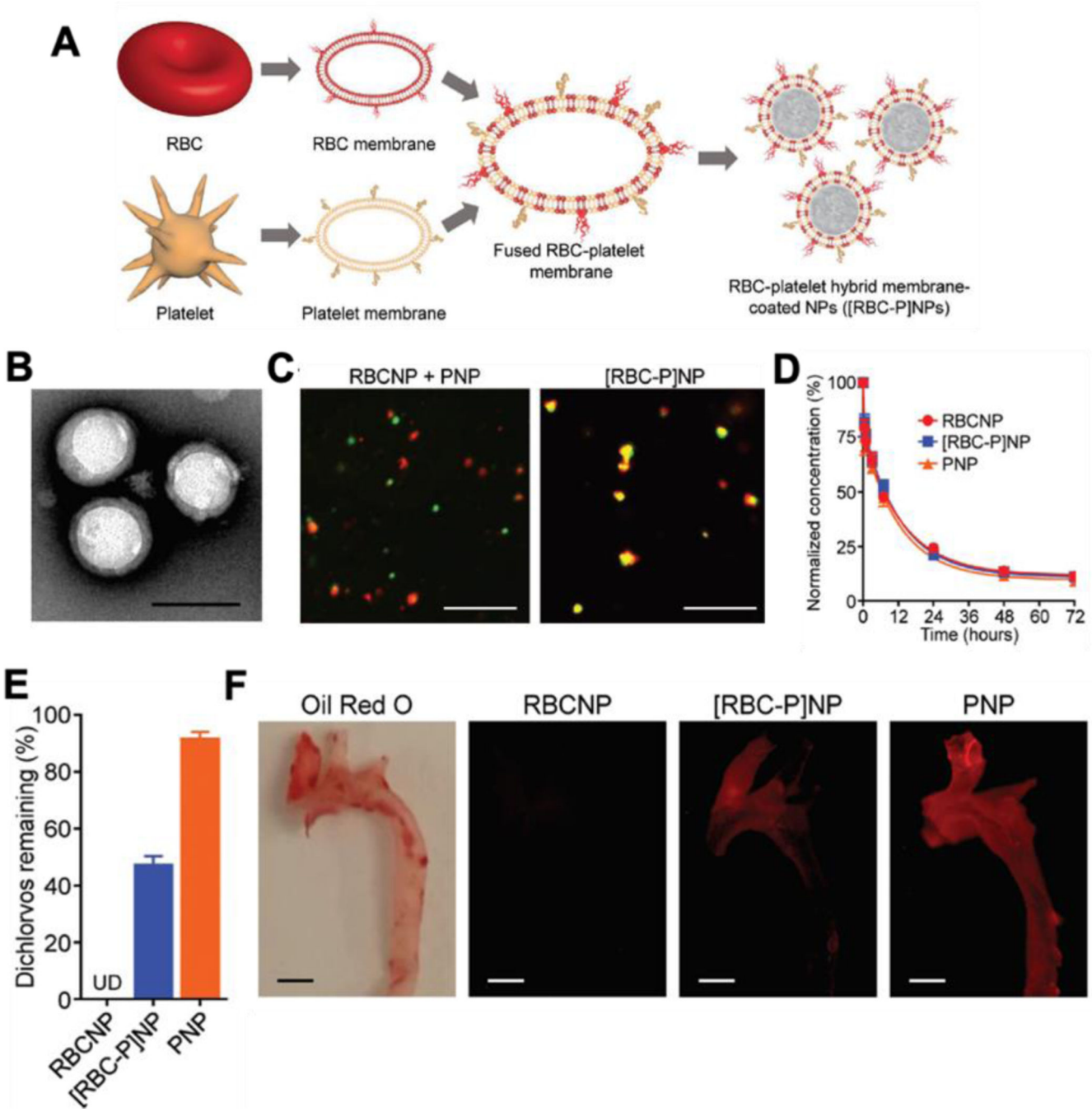


Figure 4.

Development of RBC–platelet hybrid membrane-coated nanoparticles ([RBC-P]NPs). (A) Schematic of membrane fusion and coating. Membrane material is derived from both RBCs and platelets and then fused together. The resulting fused membrane is used to coat poly(lactic-co-glycolic acid) (PLGA) polymeric cores to produce [RBC-P]NPs. (B) A representative TEM image of [RBC-P]NPs negatively stained with vanadium (scale bar = 100 nm). (C) Confocal fluorescent microscopy images of either a mixture of RBC membrane-coated nanoparticles (RBCNPs) and platelet membrane-coated nanoparticles

[PNPs] or of the [RBC-P]NPs (red = RBC membrane, green = platelet membrane; scale bar = 10 μm). (D) Circulation time of fluorescently labeled RBCNPs, [RBC-P]NPs, and PNPs after intravenous administration to mice via the tail vein (n = 4; mean ± SEM; lines represent two-phase decay model) (E) Amount of free dichlorvos, a model organophosphate, remaining in solution after incubation with RBCNPs, [RBC-P]NPs, or PNPs (n = 3; mean ± SD). UD = undetectable. (F) Imaging of aortas from ApoE knockout mice fed with a high fat western diet, after intravenous administration with dye-labeled RBCNPs, [RBC-P]NPs, and PNPs (red = nanoparticles; scale bars = 1 mm). Oil Red O staining was used to confirm the presence of atherosclerotic plaque. Reproduced with permission from ref 53. Copyright 2017 John Wiley & Sons.

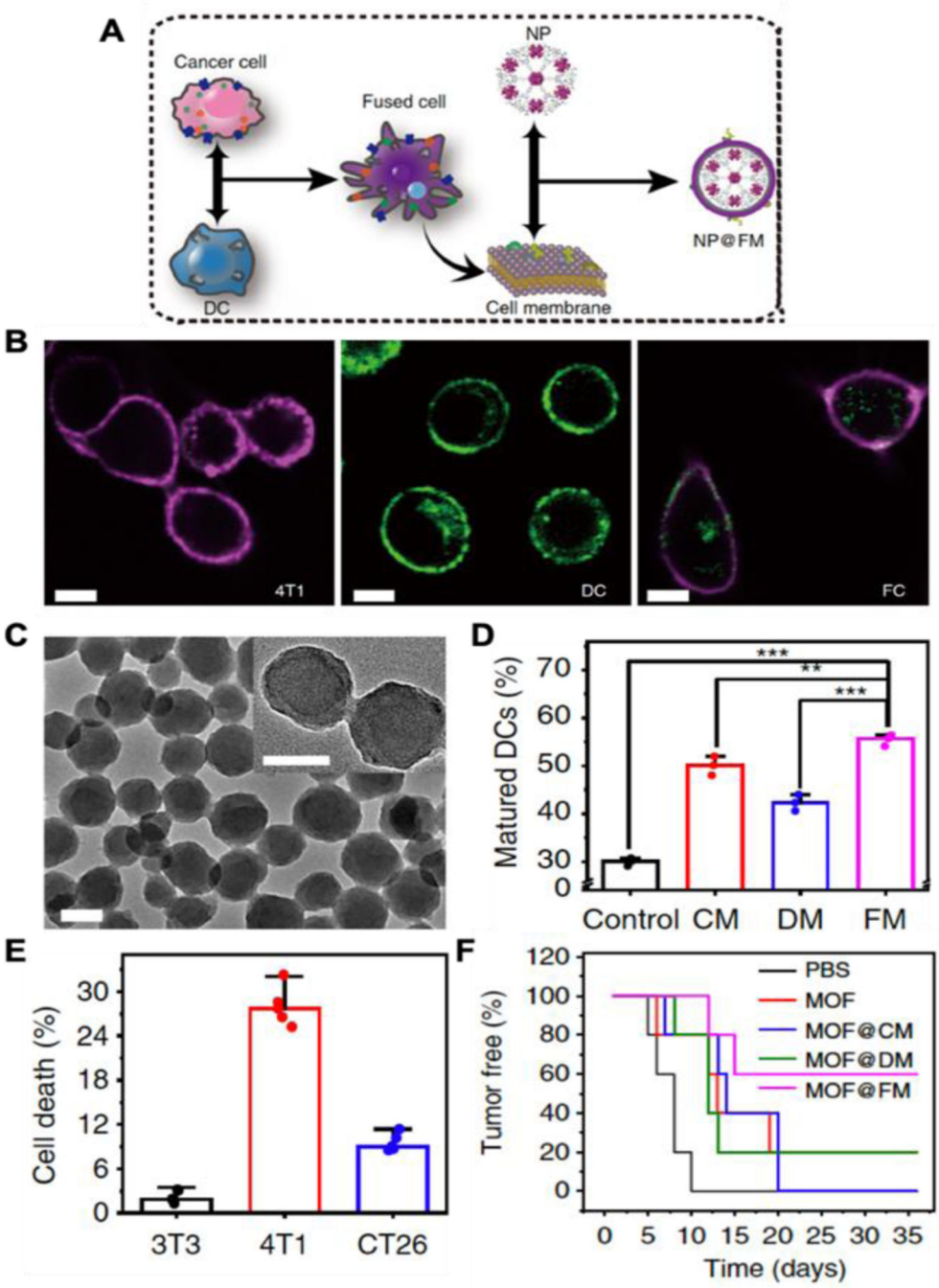


Figure 5.

Development of cancer-dendritic hybrid membrane-coated metal organic framework (MOF) nanoparticles as a cancer vaccine. (A) Schematic of the process for preparing MOF nanoparticles coated with the membrane of the fused cells (MOF@FM). (B) Dendritic cells (DCs, anti-MHC II-labeled, green), 4T1 cells (anti-CD44-APC labeled, magenta), and the fused cells (FC, double labeled) observed with the confocal laser scanning microscopy (CLSM). Scale bar = 10 μm. (C) TEM images of MOF@FM. Scale bar =100 nm. (D) Percentage of DC maturation based on the quantification of CD80 and CD86 expression

after *in vitro* incubation of DCs with 4T1 cancer cell membrane (CM), DC membrane (DM), and fused cell membrane (FM) for 48 h. The mean values and s.d. were presented and measurements were taken from distinct samples (one-way ANOVA; **p<0.01, ***p<0.001, n = 3). (E) *In vitro* cytotoxicity of the T lymphocytes after incubation with above-pretreated DCs for 48 h to 3T3, 4T1, and CT26 cells. The mean values and s.d. were presented and measurements were taken from distinct samples (n = 5). (F) Percentage of tumor-free mice receiving immunization with MOF@FM vaccine followed by tumor challenge. Reproduced with permission from ref 56. Copyright 2019 Springer Nature.

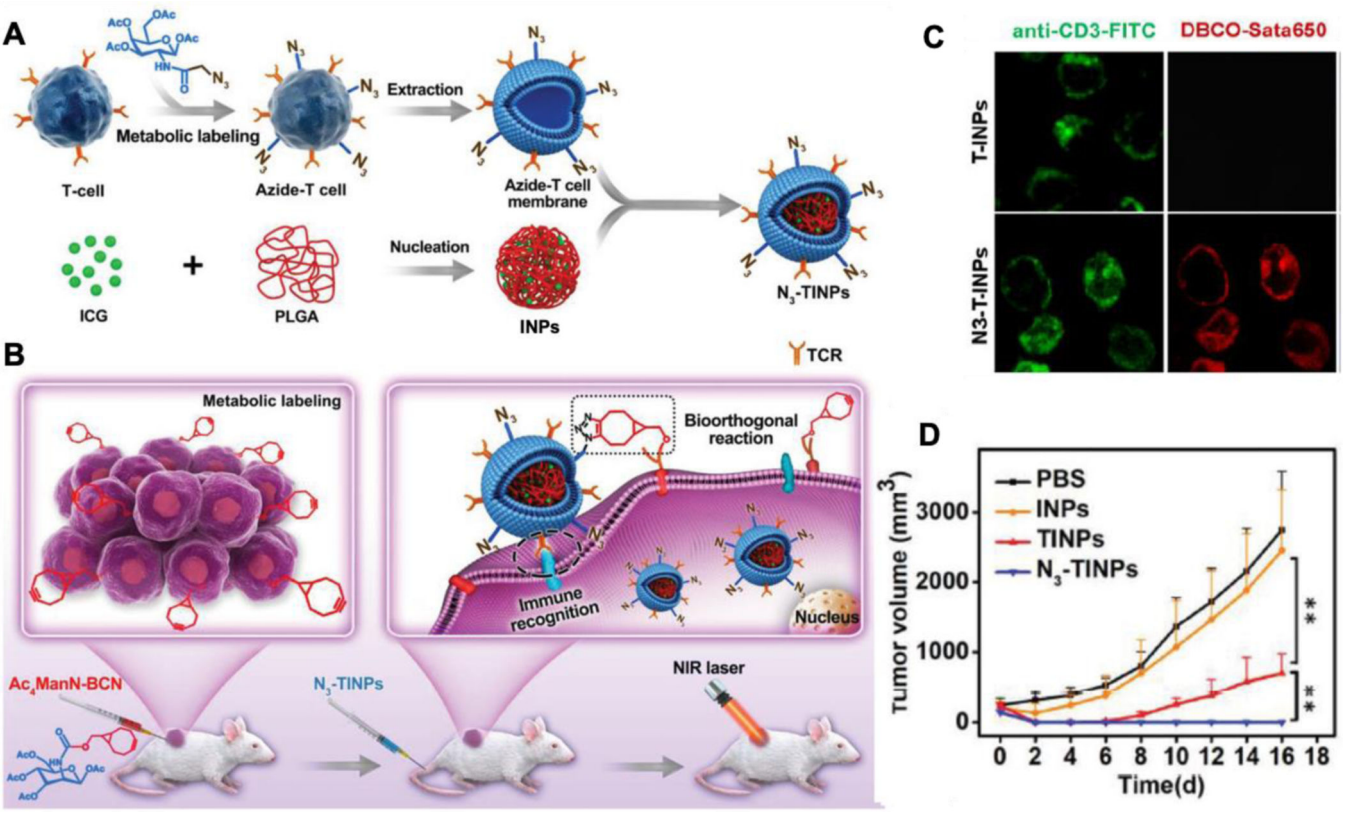


Figure 6.

Metabolic glycoengineering approach for membrane modification. (**A**) Scheme of glycoengineered T cell membrane extraction and N_3-labeled membrane-coated nanoparticles (N_3-TINPs) construction. (**B**) Illustration of tumor-bearing mice with BCN group expression upon Ac_4ManN-BCN injection. N_3-TINPs could targeted anchor in tumor region through immune recognition of T cell membrane and bioorthogonal reaction between BCN and N_3 groups, and effectively eliminate tumors based on ICG-mediated photothermal effects. (**C**) Identification of N_3 group on the surface of N_3-TINPs. Tumor cells were incubated with N_3-TINPs or TINPs (control) for 1 h, and then stain with anti-CD3-FITC and DBCO-Sata650. (**D**) *In vivo* photothermal therapy efficacy based on tumor growth curves of different groups in Raji tumor-bearing mice ($n = 5$). Reproduced with permission from ref 90. Copyright 2019 John Wiley & Sons.

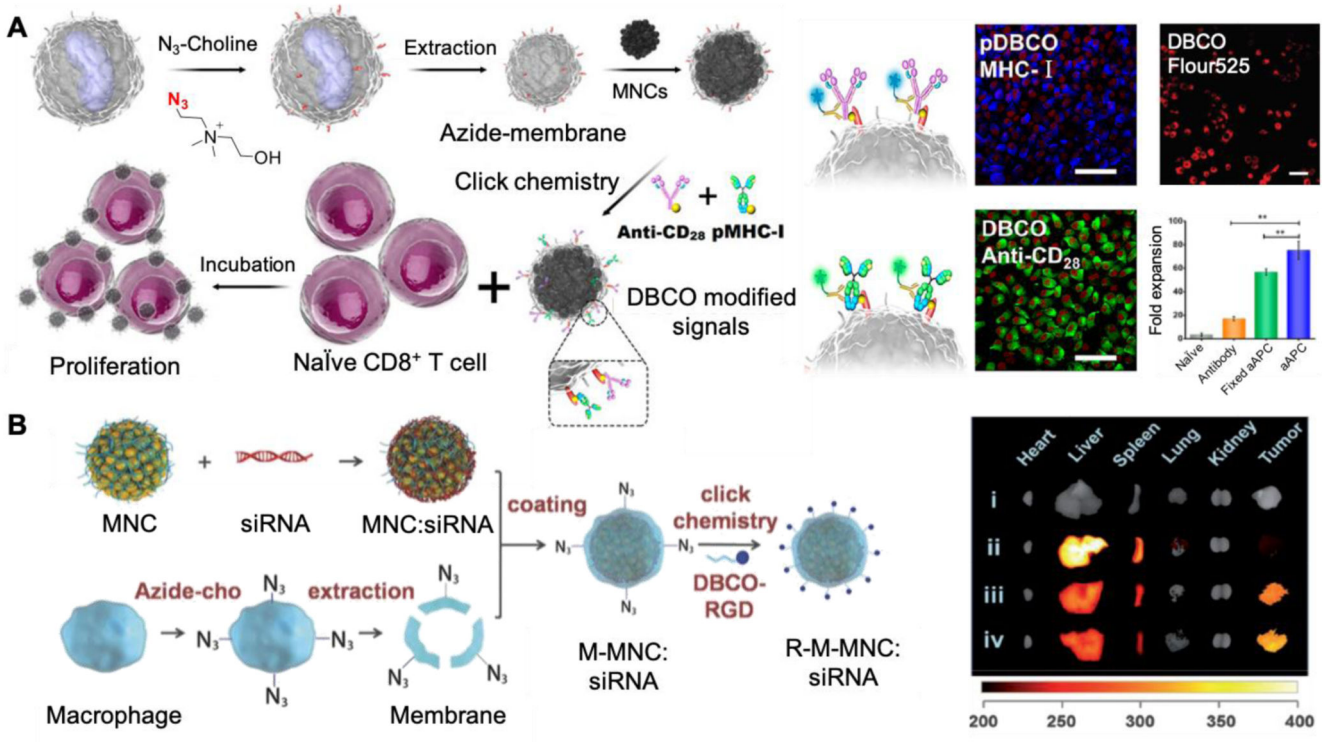


Figure 7.

Metabolic lipid-engineering approach for membrane modification. (**A**) Illustration of N_3-tagged leukocyte membrane *via* lipid-engineering to develop biomimetic nanoplatform (MNCs) for enhanced $CD8^+$ T cell proliferation. The T-cell stimuli conjugations were identified by immunostaining with the fluorescence-labeled secondary antibody of antiCD28 and pMHC-I. Then the N_3 groups on cell membrane were confirmed by DBCO-Flour525. After incubation with $CD8^+$ T cells for 7 days, the aAPCs presented the highest proliferation efficiency. Reproduced with permission from ref. [91]. Copyright 2017 American Chemical Society. (**B**) Scheme of N_3-labeled macrophage membrane-coated nanoplatform for targeted siRNA delivery. Following the modification *via* metabolic lipid-engineering, the N_3-labeled membrane was coated onto MNC-siRNA nanocomplex and conjugated with DBCO-RGD for tumor targeting. The imaging of tumor and various organs were performed at 24 h after intravenous injection of different MNC-based nanoformulations. (i) PBS, (ii) MNC:siRNA, (iii) M-MNC:siRNA, (iv) R-M-MNC:siRNA. Reproduced with permission from ref. 92. Copyright 2018 John Wiley & Sons.

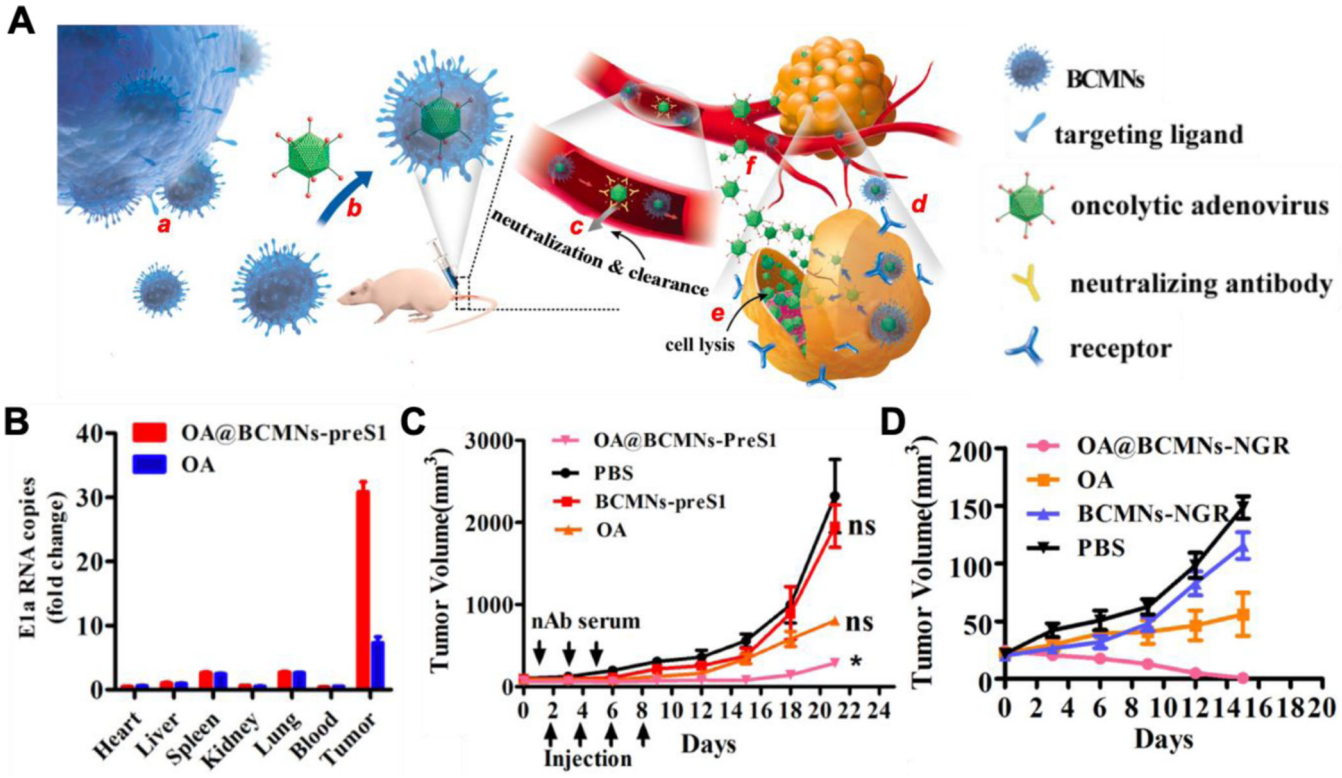


Figure 8.

Schematic of bioengineered cell membrane nanovesicle coated oncolytic adenoviruses (OA@BCMNs) for OA delivery and *in vivo* antitumor efficacy of OA@BCMNs. **(A)** Design features and proposed mechanism of OA@BCMNs. The BCMNs encapsulated OA, protecting OAs from neutralizing antibodies and delivering them to tumors through receptor mediated endocytosis. Once entered tumor cells, OAs infect and amplify the tumor cells, causing the tumor cell lysis. **(B)** Viral genome copies in excised tumors and organs, after intravenous injection of OA and OA@BCMNs-preS1 into HepG2-NTCP bearing nude mouse model, were quantified using real time qPCR. **(C)** Tumor growth curve of HepG2-NTCP bearing nude mouse model after the indicated treatment. **(D)** Tumor growth curve of HepG2-APN bearing mouse model after the indicated treatment. Reproduced with permission from ref 113. Copyright 2019 American Chemical Society.

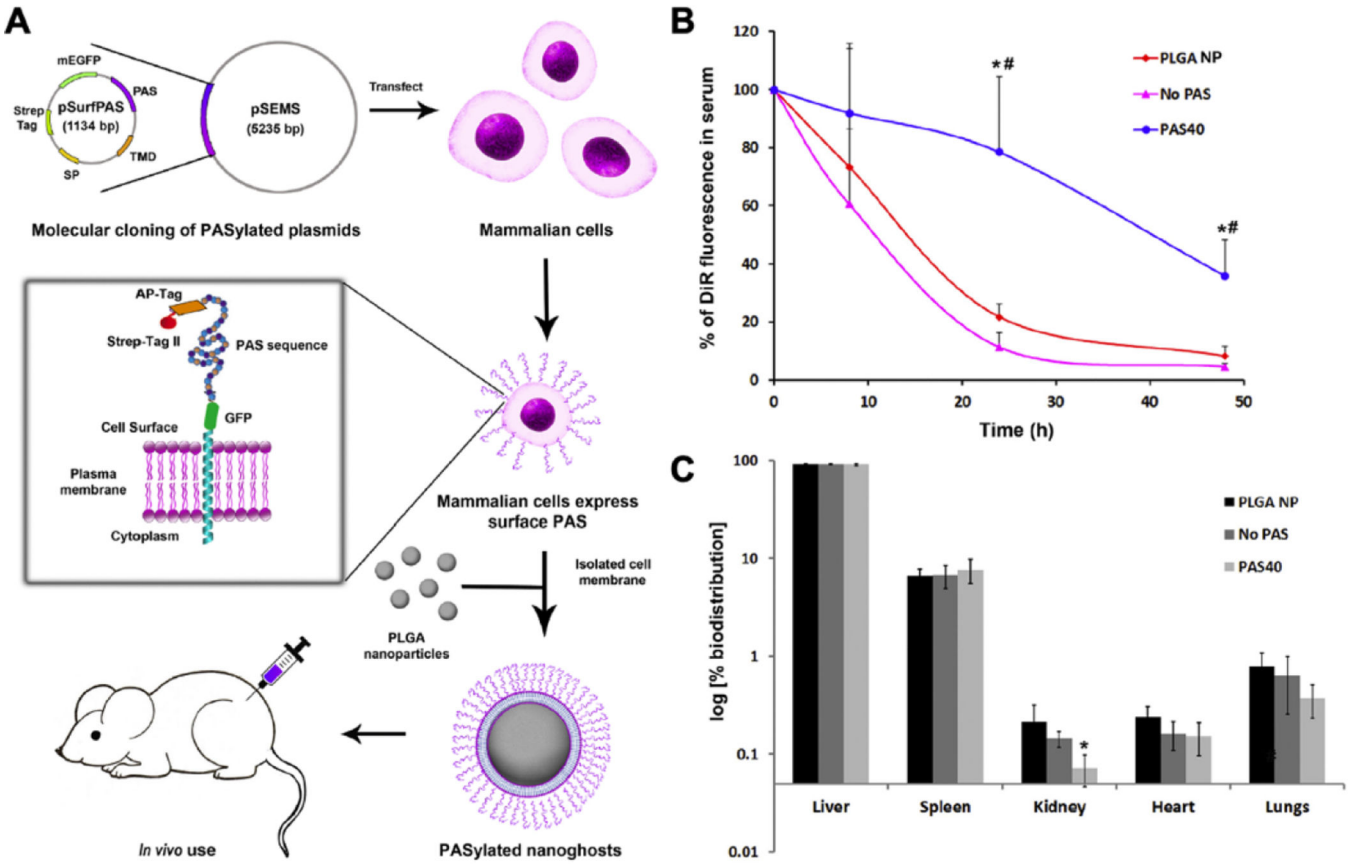


Figure 9.

(A) Illustration of the steps involved in the synthesis of PASylated nanoghosts. A plasmid that expresses the proline-alanine-serine (PAS) peptides on the surface membrane is transfected into mammalian cells. PASylated cell membranes are then harvested and coated on PLGA nanoparticles (PLGA NPs) to form PASylated nanoghosts. (B) *In vivo* serum concentration of DiR dye from nanoparticle groups over 48 h. Sample groups are PLGA NPs, non-transfected nanoghosts (No PAS), and PASylated nanoghosts (PAS40). ∗ and # denote statistical significance of PAS40 ($P \leq 0.005$) in comparison to No PAS and PLGA NP. (C) Biodistribution of dye-loaded sample groups in the liver, spleen kidney, heart and lungs of mice at 48 h post treatment. * denotes statistical significance ($P \leq 0.001$) in comparison to the PLGA NP control group. Reproduced with permission from ref 115. Copyright 2019 Elsevier.

Table 1.

Functionalization of cell membrane-coated nanoparticles by lipid insertion

Ligand	Spacer	Membrane source	Target cell (receptor) and additional function	References
Small molecules				
AS1411 aptamer	PEG2000	RBC	breast cancer cell (nucleolin)	25
Folate	PEG2000	RBC	cervical cancer cell (folate receptor),	25
			ovarian cancer cell (folate receptor)	27, 29
			breast cancer cell (folate receptor)	28, 30, 40
Mannose	PEG2000	RBC	antigen presenting cell (mannose receptor)	34
		cancer cell	dendritic cell (mannose receptor)	38
cRGD	PEG2000	RBC	melanoma cell ($\alpha_v\beta_3$ integrin)	35, 37
Angiopeptide 2	PEG2000	RBC	glioblastoma cell (LRP receptor)	33, 36
Stroke homing peptide	PEG2000	RBC	apoptotic neuron cell	31
T7/NGR peptide	PEG2000	RBC	brain endothelial cell (transferrin receptor), glioblastoma cell (CD13)	32
Biotinylated CDX peptide	PEG3400-streptavidin	RBC	brain endothelial cell (nAChR)	50
Biotinylated c(RGDyK)	PEG3400-streptavidin	RBC	tumor vasculature endothelial cell, glioma cell ($\alpha_v\beta_3$ integrin)	49
Antibodies				
Anti-HER2	PEG2000	RBC	ovarian cancer cell (HER2)	44
Anti-EGFR-iRGD	PEG3400	RBC	gastric cancer cell (EGFR, $\alpha_v\beta_3$ integrin)	41
	n.a.	RBC	colorectal cancer cell (EGFR, $\alpha_v\beta_3$ integrin)	42
Biotinylated anti-EpCAM	PEG2000-biotin-avidin	RBC	breast cancer cell (EpCAM)	43
Anti-TGFβRII	PEG2000-azobenzene	Cancer cell	hypoxia-triggered release of TGFβ-neutralizing antibody	39
Lipid with responsive functions				
DiR	n.a.	RBC	NIR-triggered membrane disruption for drug release	51
PEOz	n.a.	Platelet	pH-sensitive membrane disruption for drug release	52

Abbreviations: LRP—low-density lipoprotein receptor-related protein, nAChR—nicotinic acetylcholine receptor, n.a.—not applicable

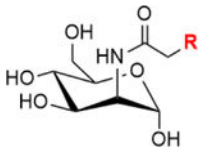
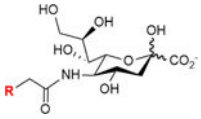
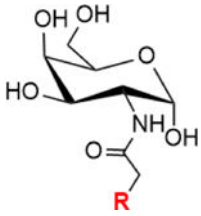
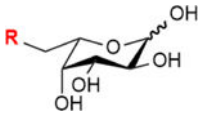
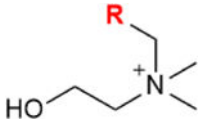
Table 2.

Summary of the studies that made hybrid membranes to functionalize nanoparticles

Membrane	Functionalities	Additional membrane	References
RBCs	Provide markers of self, neutralizing pore-forming toxins	Platelets	53, 55, 62
		Cancer cells	54, 66
Platelets	Provide markers of self	RBCs	53
		Cancer stem cells	67
	Offer ligands for targeting circulating tumor cells (CTCs)	Leukocytes	58
		Neutrophils	63
Leukocytes	Confer homologous features to reduce the unintended cell-binding interactions	Platelets	58
Macrophages	Provide markers of self	Cancer cells	65
	Confer homologous features to reduce the unintended cell-binding interactions	Cancer cells	68
Neutrophils	Offer ligands for targeting CTCs	Platelets	63
Dendritic cells (DCs)	Provide immunological co-stimulatory molecules and lymph node-targeting	Cancer cells	56, 57
Cancer cells/Cancer stem cells	Offer homotypic targeting to tumors and CTCs	RBCs	54
		Macrophages	65, 68
		Dendritic cells	56, 57
		Platelets	67
Bacterium (Salmonella)	Serve as an immunological adjuvant to induce DC maturation	Cancer cells	69

Table 3.

Summary of metabolic engineering approaches used for functionalizing cell membrane-coated nanoparticles

Metabolic Approaches	Biosynthesis Pathways	Metabolic Substrates	Chemical Structures
Glycoengineering	Sialic acid pathway	ManNAc	
	Sialic acid pathway	Neu5Ac	
	GalNAc salvage pathway	GalNAc	
	Fucose salvage pathway	Fucose	
Lipid-engineering	CDP-choline pathway	Choline	

*Note: GalNAc:*N*-acetylgalactosamine, ManNAc:*N*-acetylmannosamine, Neu5Ac:*N*-acetylneuraminic acid, CDP-choline: cytidine 5′-diphosphocholine. R= azide, alkynes, alkenes, ketone, thiol, isocyano, and diazirine groups.*

Table 4.

Summary of common transfection methods for gene delivery

Category *(selective examples)*	**Pros**	**Cons**
Viral based *(lentivirus, adenovirus, adeno-associated virus)*	• High transfection efficiency. • Easy to produce and use.	• Risks of mutagenesis and immunogenicity • Limited space for packing the genomic materials.
Chemical *(cationic lipid, cationic polymers)*	• Easy to use and can be produced in large scales. • efficiency *in vitro*. • High capacity of packing genomic materials.	• *In vitro* transfection efficiency varied by cell types. The in vivo efficiency is low. • Potential cytotoxicity depends on lipid or polymer used. • Lack of target specificity.
Physical *(electroporation, laser-irradiation, gene gun, microinjection)*	• High *in vitro* transfection efficiency, regardless of cell type. • Can achieve single-cell transfection	• Need special instrument and training. • Most physical transfection methods cannot be applied *in vivo*.