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Engineering antiphagocytic biomimetic drug carriers

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Abstract

Drug-delivery carriers have the potential to not only treat but also diagnose many diseases; however, they still lack the complexity of natural-particulate systems. Cell-based therapies using tumor-targeting T cells and tumor-homing mesenchymal stem cells have given researchers a means to exploit the characteristics exhibited by innate-biological entities. Similarly, immune evasion by pathogens has inspired the development of natural polymers to cloak drug carriers. The ‘marker-of-self’ CD47 protein, which is found ubiquitously on mammalian cell surfaces, has been used for evading phagocyte clearance of drug carriers. This review will focus on the recent progress of drug carriers co-opting the tricks that cells in nature use to hide safely under the radar of the body’s innate immune system.

Chemotherapy drugs are notorious for having poor solubility, poor stability and unwanted toxicity. To overcome these and other barriers, nanoparticles of different morphologies, functionalities and compositions have been investigated for use in nano-oncology [1–4]. Incorporation of chemotherapeutic agents into nanoparticle- based delivery systems, such as liposomes, polymeric nanoparticles, nanocapsules and micelles, has been studied extensively for the delivery of drugs to treat a wide range of cancers [5–7]. Development of a good drug-delivery system relies heavily on the selection of appropriate design parameters, such as physiochemical limitations of the drug and/or route of administration. The development of nanoparticles has evolved substantially over the last few decades and there are several key strategies in the design of nanoparticles for therapeutic applications [8,9]. For example, for a drug to be effectively absorbed, the drug must be administered in an appropriate manner. The choice of an appropriate administration route is dependent upon several factors including the type of drug chosen, carrier being used and which initial biological barriers need to be overcome. Route of administration can be broadly categorized into local administration (i.e., topical treatment) and systemic (i.e., enteral and parenteral) [10].

The success of **therapeutic carriers** is reliant upon the mode of administration. For example, administration of a drug carrier in the enteral route is characterized by absorption of the drug through the GI tract and includes oral or rectal administration. By contrast, parenteral drug administration does not pass through the GI tract but is administered directly into the bloodstream. Each route of administration plays a marked role in the synthesis of a therapeutic carrier. For instance, oral administration must address carrier stability in the GI tract, topical treatment must offer sustained release of the drug for local action on the skin or

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in mucosal membranes, and aerosol inhalers must combat the low absorption of drugs in the solid and liquid phase. While each route of delivery has its own pros and cons, intravascular injection due to its precise and almost immediate onset of action is widely employed for drug delivery. However, intravenous injection must overcome the barriers set by the **mononuclear phagocyte system** (MPS) if prolonged blood circulation is desired. In addition, for an even more efficient delivery, carriers must be designed to specifically target the site for entry [11,12].

The size, shape and surface characteristics all play a role in the biodistribution of particles used for drug delivery. The effect of size and shape on a particle has been studied extensively [13,14]. Moreover, it is known that non-biocompatible nanoparticles are easily recognized by the MPS [15]. Ideal carriers should be between 10 and 200 nm to avoid removal by the MPS [14]. If, however, the carrier is larger than 200 nm, several strategies have been developed by researchers to prolong circulation time of the particles. The first is to pre-inject decoy carriers followed by injection of carriers containing therapeutic agents [16]. As a result, phagocytes of the MPS are saturated with decoy carriers, allowing therapeutic carriers to escape and reach the desired sites. Second, alterations to the carrier surface can reduce the rate of protein opsonization or avoid complement activation. One such way to overcome the physiochemical and biological obstacles of drugs and carriers is through **PEGylation** [17]. PEGylation is the process by which PEG chains are attached to a molecule or carrier, and the stealth properties of PEG lent to them. Since PEGylation was first described, several conjugation strategies have been developed. The variety of modification procedures offered allows for the PEGylation of a wide variety of carriers and molecules [18]. PEGylated dendrimers [19], liposomes [20,21] and micelles [22,23] have been developed as viable therapeutic agents for cancer therapy. Moreover, PEGylation of chemotherapy drugs [24,25], protein molecules [26] or monoclonal antibodies [27], has been employed to increase circulation half-life time and reduce immunogenicity, without decreasing activity. Chemical conjugation of adenovirus vectors with PEG has also emerged as a viable strategy to improve the safety and efficiency of viral gene-delivery vectors [28]. Polymers based on *N*-(2-hydroxypropyl) methacrylamide have demonstrated particular promise not only as drug carriers, but for surface coating of viruses or targeted drug carriers [29,30]. While PEGylation has improved the pharmacokinetic properties of the aforementioned systems, overcoming immune clearance and cell entry across the endothelial lining and into the tissue itself is still something that needs to be addressed [31]. Furthermore, PEG has several drawbacks such as hypersensitivity reactions, non-biodegradability, instability under stress and toxicity of side products [31]. These disadvantages have prompted interest into the investigation of alternatives to PEG (Figure 1). There have been several alternatives to PEG suggested that reduce opsonization of drug carriers and increase blood circulation time [3,32,33].

Evasion of immune surveillance by nature

While synthetic biomaterials for therapeutic delivery have been significantly advanced in terms of functionality and diversity, they still lack in the complexity of natural particulates. For example, red blood cells (RBCs) exhibit a complex biological functionality: they possess a small size, flexibility and chemical composition, which allow them to complete complex tasks flawlessly in the human body without being recognized as foreign particles for up to 4 months [34]. Pathogens, such as bacteria and viruses, have developed immune-escaping mechanisms that allow them to infect tissues with maximum efficacy [35]. Recently, researchers' understanding of immune surveillance has been growing greatly [36–38], and several immune system components have been implicated in the identification and elimination of cancer cells [39,40].

Erythrocytes

RBCs exhibit all of the properties that researchers desire as an ideal drug carrier. RBCs have a flexible structure, unique shape and small size, which allow them to pass through narrow capillary networks and avoid sequestration by MPS organs such as the spleen and liver. To exploit the use of RBCs as delivery carriers, researchers are loading natural RBCs with therapeutic agents without compromising the biological functions of RBCs or their structural integrity [41]. RBCs have a semi-permeable membrane, which offers sustained release of small-molecule drugs yet retention of large proteins [42]. In addition, advances in molecular biology and bioengineering have provided scientists with a greater understanding of the physiochemical characteristics and biological function of RBCs. RBCs are produced through erythropoiesis, the process by which a committed stem cell matures into an erythrocyte. Once matured, the physiochemical and surface properties of RBCs allows for blood circulation for about 120 days before entering their graveyard – the spleen.

In 2000, Oldenborg *et al.* reported that **CD47** functioned as a ‘marker-of-self’ on murine RBCs [43]. Murine RBCs deficient in CD47 were rapidly cleared from the bloodstream by macrophages compared with wild-type mice. Indeed, as a RBC ages, CD47 expression levels decrease (30% lower expression of CD47) on the oldest RBCs compared with the youngest RBCs, clearly supporting the role of CD47 in longterm circulation of RBCs in the bloodstream [44]. CD47 is widely expressed on a majority of normal tissues in humans and other mammals, and leads to the phagocytosis of aged or damaged cells [45]. CD47 regulates phagocytosis by its interaction with SIRP α on macrophages [46]. Through CD47 ligation with SIRP α , tyrosine phosphatase is activated and myosin accumulation at the phagocytic synapse is repressed, leading to inhibition of Fc γ and complement receptor mediated phagocytosis [47]. If the inhibitory signals generated by CD47–SIRP α association are sufficient to counteract the phagocytic signals mediated through Fc γ and the complement receptors, ingestion of particles (e.g., RBCs or CD47-tagged drug carriers) is reduced or inhibited (Figure 2).

Additional circulating body cells

The body’s healthy cells are generally ignored by immunosurveillance until the onset of antigen expression for cleaning up. Hence, successful delivery of therapeutic agents to target sites in the presence of a hostile environment (e.g., neutralizing antibodies, complements and the MPS) can be achieved by hiding them inside endogenous circulating cells. T lymphocytes use the circulatory system to move between lymphatic organs and sites of foreign antigen expression, and thus tumor-targeting T cells can act as cell carriers for systemic delivery. As an alternative to antitumor T cells, tumor-homing cytokine-induced killer (CIK) cells obtained by *ex vivo* treatment of human peripheral blood lymphocytes are also potential cell carriers.

Mesenchymal stem cells (MSCs) are attractive candidates as cell carriers for several reasons. MSCs can be isolated from a variety of human tissues and organs, such as the brain, liver, kidneys, lungs, bone marrow, muscle, pancreas and umbilical cord, among others [48,49]. Specifically, MSCs derived from bone marrow possess the ability to differentiate into several lineages as well as demonstrate the highest degree of plasticity (one cell type transdifferentiated into other types) [50]. Following re-implantation, MSCs have been found to suppress the immune system and re-integrate into tissue architecture [51]. A plethora of studies have reported that MSCs migrate to sites of injury, ischemia and tumor microenvironments. The tropic mechanisms by which MSCs migrate across endothelium and home to the target tissues are associated with various cytokine/receptor pairs [52]. Moreover, MSCs can be modified by gene vectors *ex vivo* to express and release therapeutic factors [53].

Tumor cells

The immune system is distinguished by its ability to differentiate between markers-of-self and non-self. Every cell in the body carries a distinctive set of surface proteins – known as MHC complex proteins, which normally, immune cells will not attack. While it is known that the adaptive immune system plays an important role in antitumor immunity, it has only recently been discovered that the innate immune system could be an important component as well. Since the discovery that natural killer (NK) cells eliminate target cells that do not express markers-of-self [54], a wealth of information on activation and inhibition of the receptor–ligand interactions on NK cells has been discovered [55].

Several innate cells have been actively associated with antitumor immune response. Due to inflammatory signals induced by tumor cells, NK cells, T cells, macrophages and dendritic cells are recruited to the tumor site and stimulate the production of IFN- γ , thus inducing some tumor death and promoting the production of chemokines [36]. Recently, quite a few patient-derived tumor cells were analyzed with high expression of the surface protein CD47 [56–58]. It has been demonstrated that evasion of phagocytosis through upregulation of the antiphagocytic antigen CD47 is a mechanism by which tumor cells escape innate immune surveillance [59]. This happens by the interaction between CD47 and SIRP α . After binding of CD47 to SIRP α , signals are transmitted from SIRP α to the macrophage and lead to inhibition of the phagocytic process. For survival and metastasis of tumor cells, it is of pivotal importance for the cancer cells to avoid elimination by tumor-infiltrating phagocytes. Therefore, the highly expressed CD47 on tumor cells has been recently studied. The CD47–SIRP α interaction can be therapeutically targeted with a monoclonal blocking antibody against CD47, which enables phagocytosis of acute myeloid leukemia, non-Hodgkin's lymphoma, breast cancer, colon cancer, glioblastoma and ovarian cancer cells [56,57]. Furthermore, it was demonstrated that anti-CD47 antibody can synergize with other therapeutic antibodies (e.g., rituximab and trastuzumab) to eliminate cancer cells [56,58].

Pathogens

The immune system is the first line of defense when it comes to defending the host from infection by other organisms or foreign objects. If a foreign object or pathogen cannot be neutralized by the innate immune system, the adaptive immune system will be triggered. Still, pathogens that rapidly evolve and adapt to their environments can pass the scrutiny of the host immune system in a number of ways. A multitude of genes and their expressed proteins on pathogens have been found to interfere with macrophage signaling and result in phagocyte sabotage [60]. For example, *Yersinia* bacteria are some of the best characterized with an ability to interfere with phagocytosis. The virulence factor proteins are delivered into macrophages and interrupt the signaling required for phagocytosis by targeting several cytoskeletal proteins [61]. In particular, the Yad A proteins of *Yersinia enterocolitica* bind to factor H, which contributes to pathogenicity [62]. The staphylococcal complement inhibitor from *Staphylococcus aureus* blocks complement activation pathways by binding specifically to activator-bound convertases, but not to free components. Following binding, surface-bound C3 convertase are stabilized and enzymatic activity is inhibited [63].

Some pathogens can hide their antigenic surface by covering their surface with capsular polysaccharides. As a result, phagocytes cannot recognize pathogens upon contact and the possibility of opsonization by antibodies to enhance phagocytosis is diminished. For example, *Pseudomonas aeruginosa*, found in the lungs of patients with cystic fibrosis, displays hydrophilic polyuronic acid polysaccharide, which interferes with phagocytosis [64]. Group A *Streptococci* express a capsule composed of a homopolymer of hyaluronic acid, which prevents phagocytic ingestion and killing [65]. Capsule-deficient group A *Streptococci* produced by targeted mutagenesis of the hyaluronic acid biosynthetic operon

become susceptible to phagocytic clearance [66]. In addition, pathogens can deposit sialic acid residues on their surface to prevent opsonization by complement components and impede recognition by phagocytes. Glycoproteins on the external envelope of HIV viruses have been reported to shield the virus from detection [67].

Biomimetic approaches used for drug carriers

Engineering a delivery system that is biocompatible, biodegradable, non-immunogenic and long circulating is desired for effective therapeutics. To this end, the remarkable properties cells in the body possess are being exploited for the delivery of therapeutic and/or diagnostic agents. Due to the increased understanding of circulating body cells as carriers, surface biochemistry of blood cells, and the immune-escaping mechanisms pathogens and tumor cells employ, researchers are taking a lead from nature in the design of drug carriers. Aided by advances in nanotechnology and biology, researchers are able to design delivery vehicles that mimic natural cells. These novel **biomimetic** strategies have been proposed to camouflage drug carriers in order to bypass immune surveillance. These systems prolong the circulation time in bloodstream and lead to effective delivery of theranostic agents.

Erythrocyte-inspired therapeutic delivery

To develop a RBC carrier, a multitude of techniques have been established for the loading of therapeutic agents without changing the physiological survival properties of RBCs [68]. Of the erythrocyte ghost preparation methods available, hypotonic treatment is the standard preparation that retains the physiochemical characteristics of erythrocyte membranes [42,69]. RBCs are placed in a hypotonic solution where osmotic pressure releases the intracellular contents within the RBC membrane. Meanwhile, the therapeutic agent of interest in the solution can then passively diffuse into RBCs and retain inside the cells via isotonic buffer resealing (Figure 3A). Due to the pioneering studies using RBCs to deliver β -glucosidase and β -galactosidase for the treatment of Gaucher's disease [41], several enzyme-replacement therapeutics have been developed, some of which have entered long-term clinical trials [70–73]. Furthermore, erythrocyte ghosts have been harnessed for the delivery of oligonucleotides [74] and plasmid DNA [75]. RBC carriers have also been used to entrap small drug molecules [76], increasing biodistribution of the drug. Likewise, the encapsulation of multiple drugs have been studied [77]. Moreover, erythrocyte ghosts have been engineered to contain the hemagglutinin fusion protein on lipid membranes, superparamagnetic nanoparticles and the anticancer drug, decitabine, for sustained and targeted delivery [78].

RBC membrane-derived liposomes, termed nano-erythroosomes, were first reported by Lejeune *et al.* [79]. In their study, daunorubicin-conjugated nano-erythroosomes had greater antineoplastic index than the free drug. Recently, Hu *et al.* used the plasma membrane of erythrocyte ghosts to cloak biodegradable polymeric nanoparticles via membrane extrusion, and demonstrated extended blood circulation half-life time of RBC membrane-camouflaged polymeric nanoparticles (Figure 3B) [80]. Compared with PEG-coated nanoparticles, which had an elimination half-life of 15.8 h, the RBC membrane-coated nanoparticles had an elimination half-life of 39.6 h, nearly double that of the control. While these results provide opportunities in the field of drug delivery for translational medicine, many challenges still remain. Risk of rejection is possible if immunogenic species are not removed during fabrication steps. In addition, surface antigens to specific blood groups need to be addressed to optimize drug carriers for clinical use.

Another way in which researchers are taking advantage of the properties of RBCs is through the coupling of therapeutic agents onto the outer surface of RBCs by covalent conjugation [81]. Taking advantage of ligands on the RBC surface, such as the complement receptor 1,

drugs can be conjugated and their circulation time increased. A ‘hitchhiking’ strategy was employed to attach therapeutic polymeric nanoparticles on the surface of RBCs through physical means and demonstrated a drastic improvement in blood circulation time [82]. Moreover, polymeric nanoparticles attached onto RBCs did not affect the circulation of RBCs. One foreseeable challenge with drug or nanoparticle attachment onto the membrane of RBCs is the capability of sustained drug release. Increasing binding strength will vastly improve control over a therapeutic agent’s release. In addition, the particles associated with the surface of RBCs could be physically detached because of high shear stress in the bloodstream or significant RBC deformation in capillaries. In an effort to mimic the mechanobiological characteristics of RBCs, several groups have synthesized man-made vehicles. Doshi *et al.* synthesized RBC-like particles that mimic the capabilities of RBCs, such as the ability to carry oxygen and flow through capillaries smaller than their own diameter [83]. Merkel *et al.* synthesized hydrogel microparticles with tunable elasticity that resembled RBCs [84]. Both studies demonstrated that mechanobiological mimicry of RBCs was capable and that the particles had an increase to particle deformability as well as extended circulation times.

Carrier cells for therapeutic delivery

Natural carrier cells such as CIK cells, T cells and MSCs have all been exploited for the delivery of various therapeutic agents and for the delivery of oncolytic viruses (Figure 4). An advantage of using these cells is for their tumorhoming or tumor-targeting abilities. Moreover, addition of therapeutic agents into these cells does not affect the integrity of the natural particulates. CIK cells have demonstrated the ability to localize into leukemia tissue and become toxic to the tissue [85]. CIK cells have been effective in the treatment of several solid malignancies, including those of the lungs and ovaries [86,87]. Success of CIK cell treatment *in vivo* has led to clinical studies of the treatment for solid tumors with encouraging results [88]. Several clinical trials involving autologous CIK cell treatment of myeloid leukemia and non-Hodgkin lymphoma have been completed with promising results [89,90]. Similar to CIK cells, T cell-based immunotherapies have been successful in tumor immunotherapy by increasing the numbers of endogenous NK and CD8⁺ T cells, which are potent mediators of antitumor immunity [91,92]. Recently, T cells co-transduced with prostate tumor antigens to target tumor cells were successfully used for tumor treatment by Kloss *et al.* [93]. In the study, T cells were rendered tumorspecific and elicited complete tumor remission without sacrificing T-cell reactivity.

MSCs exhibit migratory properties that make them attractive candidates for the targeted delivery of genes and viruses. Recent studies have demonstrated that MSCs are not only able to migrate to and incorporate within the connective tissue of tumors, but actively seek out metastases far removed from the primary tumor site [94–96]. MSCs have been utilized as delivery vehicles of interleukins in order to improve anticancer immune surveillance by activating cytotoxic lymphocytes and NK cells [97]. Nakamura *et al.* delivered gene-modified MSCs by the infection with an adenoviral vector encoding human IL-2 [98]. IL-2 enhanced antitumor effect and prolonged survival of tumor-bearing rats. A combined treatment of peripheral immunization using IFN- γ transduced into tumor cells, with intratumoral delivery of MSCs expressing IL-7 was conducted by Gunnarsson *et al.* [99]. They found that the combined treatment increased the density of T cells in rats, which correlated with antimetastatic effects.

MSCs have also been used as delivery agents for gene-directed enzyme prodrug therapy. Several prodrug–enzyme activation schemes are available for killing tumor cells and are being evaluated in clinical trials [100]. The approach of using MSCs to deliver a gene to a prodrug was first explored by Aboody *et al.* through the delivery of cytosine deaminase

(CD), which can be used for activation of prodrug 5-fluorocytosine to 5-fluorouracil [101]. Kosaka *et al.* evaluated the effect of MSC transfection with adenovirus carrying CD followed by treatment of 5-fluorocytosine *in vivo* [102]. Their results showed that delivery of MSCs transfected with CD resulted in significant prolongation of survival of rats with glioma cells. Fei *et al.* transfected MSCs with lentiviral vectors expressing CD with similar success in decreased tumor viability [103]. Other prodrug–gene combinations have been studied in conjunction with MSCs. Mori *et al.* transfected HSV-*tk* into MSCs and then treated glioma cells with the MSCs/*tk*, followed by treatment with ganciclovir [104]. Their results demonstrated that significant cell death occurred in glioma cells treated with MSCs/*tk* compared with non-transfected MSCs. The use of MSCs for suicide gene therapy and as targeting vehicles is proving very promising for treatment of various cancer types.

Oncolytic viruses are either natural or genetically modified viruses that can be used to infect tumor cells without harming healthy tissues [105]. Many of the hurdles oncolytic viruses encounter after intravenous administration can be overcome by hiding oncolytic viruses inside carrier cells [106]. Associating the virus with carrier cells would then allow the carrier cells to chaperone the oncolytic virus to the tumor sites. Several cell types have shown promise in preclinical models, including T cells, dendritic cells and MSCs. Ilett *et al.* employed T cells and dendritic cells to achieve systemic delivery to tumor-targeted reoviral therapeutics [107]. In experiments, T cells and dendritic cells were used as carriers for reovirus. Results showed that antitumor immune responses and long-term clearance was achievable to melanoma tumor-bearing mice that had pre-existing antiviral immunity. MSCs have been used for the delivery of oncolytic adenoviruses. Garcia-Castro *et al.* found that ICOVIR-5, an oncolytic adenovirus, could be infused into autologous MSCs and delivered to metastatic neuroblastoma with remission for 3 years in one child [108]. The delivery of Delta24-RGD, which has shown to be effective in treatment of glioblastoma, was delivered by MSCs to mice bearing glioma tumors [109]. Through tumor imaging, tumor size decreased in MSCs with Delta24-RGD-treated animals. Additionally, median survival increased almost twofold.

The success of carrier cells in clinical studies has shown promising for tumor immunotherapy. The ease of cell expansion *ex vivo* coupled with their ability to encapsulate or express a wide range of therapeutic agents highlight carrier cells potential. Long-term safety and efficiency still needs to be evaluated to assure the use of carrier cells in the clinic.

Pathogen-inspired therapeutic delivery

As our understanding of the methods by which pathogens evade the immune system has grown, the ability to apply the **immune evasion** capabilities of pathogens to material design has been developed. One of the earliest attempts to exploit a naturally derived polysaccharide was through the coating of liposomes with linear dextran [110]. Bacterial dextran is produced by the fermentation of sucrose with *Leuconostoc mesenteroides* [111]. After ethanol precipitation from the culture medium, acid hydrolysis is used to reduce the overall MW of dextran. Dextran with an average MW of 70 kD has been used as a plasma volume expander [112]. Dextran coating enhances the circulation time of drug molecules [113] and stability of micelle carriers [114]. The effect of dextran conformation on self-assembly behavior of micelles should be conducted in more detail. Coating of poly(lactic acid) nanoparticles with dextran induced a diminished protein adsorption [115]. Again, conformation of dextran played an important role because blood circulation time was not increased and the authors attributed this to the loop conformation of the polysaccharide chains. Dextran coating prolonged the blood circulation time of carriers made of poly(methyl methacrylate) polymer [116] and contrast agents [117]. Dextrin, which is similar in structure to dextran, was self-assembled into nanoparticles by Goncalves *et al.*

[118]. In a subsequent study, the interactions of dextrin nanoparticles with murine macrophages were found not to elicit an immune response [119]. Similarly, blood circulation time was increased *in vivo*. Hyaluronan covalently attached to the surface of lipid nanoparticles has been demonstrated to endow the carriers with extended circulation and active targeting towards hyaluronan receptor CD44, which is highly expressed on tumors [120]. According to recent findings, Mizrahy *et al.* inferred that nanoparticles bearing low-molecular-weight hyaluronan could be a viable option for the replacement of PEG when passive delivery is required [121].

Heparin is known to modulate the action of several systems including viral entry [122] and angiogenesis [123]. Heparin is a member of the glycosaminoglycan family of carbohydrates and found on the surface of cells and in the extracellular matrix [124]. It is known that heparin-like polysaccharides can be obtained from microorganisms such as *Escherichia coli* and resembles a type II glycosaminoglycuronan chain [125]. Due to its biological and pharmacological activities heparin has been used to surface-functionalize a variety of nanoparticles [126]. Heparin-coated poly(methyl methacrylate) nanoparticles exhibited a prolonged blood circulation time for 2 days because host factor H can bind to heparin and inhibit complement activation [116]. Recently, a heparin-mimicking polymer conjugate was synthesized by Nguyen *et al.* [127]. The synthesized heparin was far more stable under stress than the control without sacrificing the native activity of heparin. For drug-delivery carriers this is advantageous because the synthesized heparin could be more easily tailored to suit the therapeutic agent's needs. However, whether or not the mimicked heparin can endow drug carriers with antiphagocytic function, is yet to be discovered.

CD47

After the discovery of CD47 as a marker-of-self on RBC surfaces [43], it was suggested by Moghimi *et al.* that CD47 could represent a viable solution in the design of macrophage-evading therapeutic carriers [128]. Hsu *et al.* first demonstrated the antiphagocytic effect of the soluble CD47-streptavidin (CD47-SA: containing only the extracellular domain of CD47) fusion protein, expressed in BL21 bacteria and then purified by biotinylated agarose column, on macrophage cells [129]. In the study, it was demonstrated that perfluorocarbon-based oxygen carriers were able to avoid being engulfed by J774A.1 macrophage cells for up to 2 h after cells were pretreated with soluble CD47-SA for 1 h. The CD47-SA fusion protein was further tagged on biotinylated zymosan microparticles (prepared from yeast cell wall) labeled with FITC fluorescent dye to examine the antiphagocytic efficacy of CD47 coated on biological particles with an average size of approximately 3 μm . As shown in Figure 5, the prepared CD47-SA-biotin-zymosan-FITC microparticles interacted with macrophages for 4 h, and revealed significant antiphagocytic effect. This was due to a sufficient density of CD47 ligands bound onto the zymosan particle surface via biotin-streptavidin affinity. Tsai *et al.* employed mammalian Chinese hamster ovary cells transfected with CD47-encoding plasmid (containing only the extracellular domain sequence of CD47) to produce and biotinylate the extracellular domain of the CD47 ligand [130]. Streptavidin-coated polystyrene microbeads of 2.1 μm in diameter were tagged with biotinylated CD47 with a density comparable with that on RBCs and exhibited significant phagocytosis resistance with THP-1 macrophages. Recently, work by Rodriguez *et al.* led to the development of a man-made 'self' peptide, which can inhibit phagocytosis and enhance delivery of nanoparticles [131]. Through structure biology and computer simulation, a 21-amino acid 'self' peptide fragment (shortened from the 117-amino acid extracellular domain of CD47) was synthesized. This peptide was then put onto fluorescent polystyrene nanobeads of 160 nm. Within 30 min, the beads tagged with the peptide showed four-times as many beads still in circulation compared with the control beads. In addition, delivery of paclitaxel to tumor-bearing mice with the peptide-tagged polystyrene nanobeads showed

similar tumor size reduction as delivery by the standard paclitaxel carrier (i.e., Cremophor®) without similar toxic side effects.

Conclusion

Great strides have been made in the design of engineered nanoparticles in the past several decades. Despite the use of synthetic polymers to improve pharmacokinetics and pharmacodynamics of drug carriers, synthetic systems do not match the complexity of natural cells. The development of efficient therapeutic delivery vehicles requires evasion of the MPS, localization to the target sites and sustained delivery. Inspired by the way innate biological cells from our body are able to accomplish everyday tasks in the body, the biomimetic approach to engineering delivery systems has expanded rapidly. Natural cells including RBCs, CIK cells, T cells and MSCs have all been exploited for the therapeutic delivery of genes, nucleotides, proteins and drugs to tumor cells, while still maintaining the native cells integrity. Delivery carriers based on pathogen's stealth mimicry have shown to offer a certain degree of immune evasion. Moreover, mimicking key attributes of biological carriers, such as the physical morphologies (size and shape), mechanobiological characteristics (elasticity) and self-marker proteins (CD47) prolong a carrier's circulation time in the bloodstream, and thereby improves therapeutic delivery. While the field of biomimetic engineering has expanded greatly, the understanding of delivery mechanisms used by biological carriers is yet to be fully understood. A greater understanding of these delivery mechanisms will allow researchers to develop new and improved synthetic techniques based on innate biological cells.

Future perspective

Due to advances in molecular and cellular biology, the field of biologically inspired drug carriers has expanded rapidly. As such, there are still many branches that have yet to be explored and developed. As noted throughout this review, hydrophilic polymers, erythrocytes, carrier cells, pathogens and CD47 demonstrate their potencies of evading sequestration by the MPS scrutiny. Still, a number of different possibilities exist that can be further explored for these systems.

It has been widely studied that PEG can be conjugated to biomolecules, proteins and drug carriers, imparting stealth properties to therapeutic agents and delivery vehicles. Recent accounts, however, indicate that the host immune system can recognize PEGylated particles and therapeutic agents after repeated injections [132–134]. Alternative recombinant methods mimicking the biochemical properties of PEGylation for therapeutic delivery have emerged recently to circumvent PEG-induced immunogenicity and antigenicity. The approach is to substitute PEG by hydrophilic and flexible polypeptide chains, which is achieved through genetic fusion technology. Unlike PEG, which is nonbiodegradable and polydisperse, polypeptide chains are fully biodegradable and monodisperse, with defined length and composition. Recombinant PEG mimetics, based on the natural amino acids proline, alanine and serine (PASylation), were developed by XL-protein. The non-immunological PAS peptide sequences, which adopt a stable random conformation, can be attached via genetic fusion to N-terminus and/or C-terminus of a recombinant protein, resulting in prolonged plasma half-life of biopharmaceutics [201]. In a similar approach, the genetic fusion of an unstructured hydrophilic recombinant polypeptide (XTEN) to peptides or proteins also showed enhancement in plasma half-life extension [135]. Animal studies demonstrated that the XTEN peptide sequences are safe and poorly immunogenic [136]. Recently, Amunix (CA, USA) has developed reactive XTEN genetic fusions that can be used to tailor drug carriers through chemical conjugation [202].

RBC ghosts have long been employed for carrying therapeutic agents (around the same time as liposomes were considered as drug carriers) due to their natural long-circulating capabilities in the bloodstream. Moreover, drug nanocarriers coated with RBC plasma membranes revealed extraordinary plasma half-life extension. Having prolonged blood circulation time is no doubt a prerequisite for efficient drug delivery, yet targeting therapeutic agents to diseased sites is the ultimate aim. The challenge to overcome, however, is how to functionalize RBC plasma membranes with targeting moieties, without significant compensation of membrane integrity. Since PEGylation of RBCs has been investigated for decades with sufficient knowledge [137], use of PEG or hydrophilic polymer/polypeptides as a spacer to incorporate targeting moieties onto the RBC plasma membrane could be an option to minimize any adverse alteration to the membrane. Similarly, carrier cells such as MSCs – which have long-circulating as well as tumortropic abilities – could be enhanced through gene modification and surface coatings. Drug-loaded carriers endowed with both tumor-tropic and antiphagocytic properties could potentially be developed.

The active inhibitory peptide of pathogen virulence factor proteins could also be identified and conjugated onto drug carriers, thus, leading to increased blood circulation half-life by diminishing C3b-mediated MPS uptake. Moreover, while alternative materials have been explored as a substitute for PEGylation technology, simultaneous presence of hydrophilic polymers/polypeptides (e.g., PEG, XTEN) and antiphagocytic moieties (e.g., pathogen virulence factors and self-markers) in a single drug carrier could be complementary and worthy for exploration. For example, bioactive CD47-SA or 'self' peptide can conjugate onto PEG-functionalized nanocarriers, thereby resulting in additive cloaking properties being lent to the drug carrier.

Furthermore, through the authors' studies with zymosan microparticles, it is known that CD47-SA can be attached to a biotinylated bacterial substrate revealing antiphagocytic function. Since viral and nonviral vectors for gene therapy can be biotinylated by standard chemical or metabolic approaches [138], CD47-SA fusion protein can be easily tagged onto biotinylated gene delivery vectors and prevent them from phagocytic ingestion prior to reaching targeted sites. Additionally, since CD47 is an integrin-associated protein [46], it might be employed to target integrin $\alpha_v\beta_3$, which is overexpressed on tumor-activated neovascular endothelial cells and unexpressed on mature quiescent cells lined in blood vessels [139]. In addition to serving as an antiphagocytic ligand, we speculate CD47 could also serve to target drug carriers onto abnormal endothelial cells bearing high levels of integrin $\alpha_v\beta_3$, and thereby block tumor angiogenesis.

Overall, the outlook of natural carriers being exploited for therapeutic delivery is promising. As we garner a better understanding of how the cells in our body work and how pathogens can evade the host's immune system, we can better manipulate natural carriers and their properties for the design of our own therapeutic carriers.

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Key Terms

Therapeutic carrier

Transport system that delivers drugs, genes, proteins and/or diagnostic agents for the medical treatment of a disease.

Mononuclear phagocyte system	Part of the immune system composed primarily of monocytes and macrophages, which remove dying or foreign cells from the body. Found primarily in the liver and spleen.
PEGylation	Modification of a protein, peptide or carrier by the linking of one or more PEG chains, increasing circulation time and stability of drug or carrier, and decreasing immunogenicity and antigenicity.
CD47	Integrin-associated protein that binds to the SIRP α receptor, known as a 'marker-of-self' on red blood cells.
Biomimetic	Mimicking the properties of natural materials through the use of synthetic materials.
Immune evasion	Avoidance of the immune system by pathogens and tumors.

References

Papers of special note have been highlighted as:

▪ of interest

▪▪ of considerable interest

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Executive summary

Therapeutic delivery

- Synthesis of a therapeutic carrier is dependent upon mode of administration and the physiochemical limitations of the drug.
- PEGylation is a common way researchers alter carriers, proteins or antibodies to overcome physiochemical and biological obstacles.

Evasion of immune surveillance by nature

- Red blood cells (RBCs) have a semi-permeable membrane, which offers sustained release of small drug molecules, yet retains large proteins.
- CD47 on the surface of RBCs contributes to the long-term circulation of RBCs.
- Tumor-targeting T cells and tumor-homing cytokine-induced killer cells and mesenchymal stem cells are ignored by the scrutiny of the immune system.
- Expression of CD47 on tumor cell membranes has been verified as a means to block phagocytosis of tumor cells.
- Pathogens can evade the immune system by shielding their antigenic surface with polysaccharides or by expressing virulence factor proteins, which interfere with macrophage signaling.

Biomimetic approaches used for drug carriers

- Erythrocyte ghosts have been prepared to entrap therapeutic agents without changing the long-term blood circulating feature of RBCs.
- Through both physical and chemical attachment, therapeutic agents have been attached onto or incorporated in RBCs.
- Cytokine-induced killer cells and T cells have been employed as cell carriers for antitumor treatment.
- Mesenchymal stem cells have been exploited to deliver interleukins, genes and oncolytic viruses.
- Naturally occurring polysaccharides derived from bacteria can be applied for evading the immune system.
- Recombinant extracellular domain CD47 proteins and CD47 'self' peptide have been synthesized and demonstrated an antiphagocytic feature after being tagged onto particles.

Figure 1. Delivery vehicles for therapeutic agents and the synthetic polymers used to cloak nanoparticles from phagocytic ingestion

(A) Nanocarriers used for the delivery of therapeutic agents into diseased cells. Nanospheres are typically solid polymers with therapeutics embedded into a polymer matrix. Dendrimers are repeatedly branched polymer molecules containing a cascade of branches grown from several cores. Liposomes are composed of an inner core harboring hydrophilic drugs and enclosed by a phospholipid bilayer. Micelles form spontaneously in aqueous media due to their amphiphilic nature and encapsulate hydrophobic therapeutic agents. Solid lipid nanoparticles are composed of a solid lipid core matrix solubilizing therapeutic agents and stabilized by a surfactant shell. Nanocapsules are a shell with an inner space loaded with therapeutic agents. (B) Synthetic polymers commonly used in the formation of the stealth corona on drug carriers. These polymers camouflage carriers to evade immune surveillance and thereby prolong blood circulation time for drug delivery.

PAAm: Poly(acrylamide); PHPMA: Poly(*N*-(2-hydroxypropyl)methacrylamide); PVP: Poly(vinylpyrrolidone).

Figure 2. The interaction of phagocyte receptors and an opsonized particle tagged with CD47 ligand

The interaction of SIRP α and CD47 generates an antiphagocytic signal onto the particle and competes against the phagocytic signal triggered by Fc γ -IgG binding. If the negative signal dominates in this tug-of-war, the particle gets the chance to avoid phagocytic internalization.

Figure 3. Procedures of fabricating erythrocyte ghost-mediated therapeutic carriers and their delivery to targeted cells

(A) Red blood cell ghosts obtained by hypotonic treatment can be used to harbor various therapeutic agents in soluble form via passive diffusion in an isotonic buffer or (B) trapped in nanoparticles via membrane extrusion. Due to the surface properties of red blood cells, the engineered carriers can evade detection by phagocytes (such as monocytes and neutrophils in the blood vessels, and macrophages in the resident tissues) and deliver therapeutics to targeted cells.

Figure 4. Innate biological cells used as cell carriers for the delivery of gene-expressed therapeutics or oncolytic virus to target diseased sites

(A) A therapeutic gene encoded in a viral vector, plasmid DNA complexed with a nonviral vector or an oncolytic virus are used for (B) *ex vivo* modification of the body's cells, (C) which then home to target diseased sites.

Figure 5. Antiphagocytic potency of zymosan microparticles bound with CD47 ligand and FITC fluorescent dye

Zymosan (cell wall prepared from *Saccharomyces cerevisiae*) was first labeled with FITC via isothiourea linkage, and then conjugated with biotin using NHS-biotin. The biotinylated zymosan-FITC particles were mixed with CD47-SA fusion protein to obtain CD47-SA-biotin-zymosan-FITC particles by streptavidin-biotin affinity. The collected CD47-zymosan-FITC particles were further treated with fetal bovine serum. The opsonized CD47-zymosan-FITC particles were homogenously layered on top of murine J774A.1 macrophages. For the distinction between internalized and surface-bound FITC-labeled zymosan, trypan blue was used to quench surface-bound fluorescence. The microscopic images of macrophages were treated with (A) biotinylated zymosan and (B) CD47-tagged zymosan-FITC for 4 h (scale bar = 40 µm), respectively.

Adapted from [SAWDON A, PENG C-A, UNPUBLISHED DATA].