In vivo nano-biosensing element of red blood cell-mediated delivery

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Abstract: Biosensors based on nanotechnology are developing rapidly and are widely applied in many fields including biomedicine, environmental monitoring, national defense and analytical chemistry, and have achieved vital positions in these fields. Novel nano-materials are intensively developed and manufactured for potential biosensing and theranostic applications while lacking comprehensive assessment of their potential health risks. The integration of diagnostic *in vivo* biosensors and the DDSs for delivery of therapeutic drugs holds an enormous potential in next-generation theranostic platforms. Controllable, precise, and safe delivery of diagnostic biosensing devices and therapeutic agents to the target tissues, organs, or cells is an important determinant in developing advanced nanobiosensor-based theranostic platforms. Particularly, inspired by the comprehensive biological investigations on the red blood cells (RBCs), advanced strategies of RBC-mediated *in vivo* delivery have been developed rapidly and are currently in different stages of transforming from research and design to pre-clinical and clinical investigations. In this review, the RBC-mediated delivery of *in vivo* nanobiosensors for applications of bio-imaging at the single-cell level, advanced medical diagnostics, and analytical detection of biomolecules and cellular activities are presented. A comprehensive perspective of the technical framework of the state-of-the-art RBC-mediated delivery systems is explained in detail to inspire the design and implementation of advanced nanobiosensor-based theranostic platforms taking advantage of RBC-delivery modalities.

Keywords: nano-biosensor; nanoparticles; drug delivery; red blood cells; mediated delivery

1. Introduction

A nanobiosensor is an analytical device in nanometer scales used to probe or measure biochemical substances, usually comprises a sensing element "bio-receptor" to interact with the targeted analyte and produce a detectable physical signal to be transformed by a transducer component, making it possible to convert and quantify the biological and biochemical signals through optical, electronic, thermal, or magnetic methods (Prasad, 2014). In recent years, together with the progress in the nano-technology, nanobiosensors that can be used *in vivo* are vigorously developing as they can provide real-time, rapid, and accurate analysis of physiological and pathological processes in living organisms, which has been a hot research topic in medical and ecological diagnostics (Sadovoy and Teh, 2015).

In the emerging field of nanomedicine, novel nano-agents are intensively developed and manufactured for potential biomedical applications including nano-biosensing, nano-diagnostics, and personalized therapy while lacking comprehensive assessment of their potential health risks. In recent years, the application of smart drug delivery systems (DDSs) in the field of *in vivo* biosensing

has attracted extensive attention and been widely studied to promote the clinical early diagnosis and therapy, especially early cancer screening and treatment (Argyo et al., 2014; Wang et al., 2019). Controllable, precise, and safe delivery of diagnostic nano devices and therapeutic agents to the target tissues, organs, or cells is an important determinant in every clinical approach. In recent years, inspired by the red blood cell (RBC)-mediated delivery methods, nanomaterial-based theranostic platforms for bio-sensing applications including bio-imaging at the single-cell level, advanced medical diagnostics, and analytical detection of biomolecules and cellular activities are facing new development opportunities.

At present, there are many reviews on the medical applications based on the development of new sensing techniques capable of performing very sensitive detection and quantifying certain parameters, especially based on smart nanobiosensors (Ahmed et al., 2014; Attaallah et al., 2020; Chamorro-Garcia and Merkoçi, 2016; Ghorbani et al., 2019; Maxwell et al., 2002; Prasad, 2014). Numbers of reviews have summarized the cell-based DDSs in general (Glassman et al., 2020; Jahangirian et al., 2019; Kurapati et al., 2019; Sun et al., 2017). However, currently, there are only a few of reviews place emphasis on the RBC-mediated delivery for specific application areas and the adverse effects on human health induced by the interaction of delivered sensing devices and theranostic agents with blood components.

In this review, the emphasis is placed on the nanomaterial-based *in vivo* biosensing elements for applications of bio-imaging at the single-cell level, advanced medical diagnostics, and analytical detection of biomolecules and cellular activities that benefited from the RBC-mediated DDSs. Specifically, the technical framework of the state-of-the-art developments of RBC-mediated delivery of theranostic nano devices/agents is presented. Finally, the advantages, challenges, and the development trends of nanobiosensors based on the RBC-mediated delivery are indicated to inspire the design and implementation of advanced nano-biosensing platforms taking advantage of RBC-delivery modalities.

2. The application of nanomaterials and DDSs in biosensing

2.1. The application of nanomaterials in biosensing

The revolutionary development of nanotechnology has encouraged tremendous progress in the design of *in vivo/vitro* diagnostic/therapeutic tools utilizing nanostructures with various types and materials (Anselmo and Mitragotri, 2016; Lucky et al., 2015; Wolfbeis, 2015; Zhou et al., 2015). To date, distinguished by different dimensions and geometry, diverse types of nanomaterials including nanoparticles (NPs), nanofiber, nanotubes, nanorods, and nanoribbon have been studied (Wolfbeis, 2015). Among all modalities, NPs, defined as submicroscopic particles that are between 1 and 100 nm in size, are of great scientific interest as they are essentially a connection between bulk materials and atomic or molecular structures (Khan et al., 2019). Their versatile optical and structural merits and unique surface properties have demonstrated unexpected application prospects in biological studies. For example, gold nanoparticles (Au-NPs) have excellent size-dependent optical and optoelectronic properties, which make them promising in diverse biotechnology applications (Zhou et al., 2015). Specifically, Au-NPs could not only serve as optical markers in biosensing (Kabashin and Meunier, 2007) and imaging enhancers in scanning electron microscopy (SEM) (Khan et al., 2019), but could also be applied in cancer photothermal therapy, attributing to their strong optical absorption and subsequent nonradiative energy dissipation (Abadeer and Murphy, 2016). Silicon nanoparticles (Si-NPs) are dielectric nanostructures with excellent biocompatibility and outstanding photostability that exhibit a highly efficient tunable photoemission in the visible and near-infrared (IR) frequency ranges (Kutrovskaya et al., 2017). Intriguingly, in addition to their wide application potential in photonics in the visible and near IR ranges, pure Si-NPs are also very suitable as substrate NPs for the synthesis of hybrid Si-based nanostructures. For instance, Si-based gold nanostructures combine advantageous properties of both semiconductor and metal nanostructures in one nanocomposite (Ryabchikov, 2019). These nanostructures can serve as delivery capsules of photosensitizers or as artificial enzymes assisting photodynamic therapy and other biomedical applications in biosensing,

bioimaging, and tumor diagnosis (Lucky et al., 2015; Wu et al., 2019). Integrating nanomaterials into the sensing systems as key components has facilitated the revolutionary breakthrough in smart biosensing by achieving stable and miniaturized sensing probes, enhancing the detection signals in small sample volumes, and improving the multi-channel detection ability of the sensing systems, thus helps to achieve major breakthroughs in this field (Yüce and Kurt, 2017). Biosensing systems that based on the electrical, optical, and electrochemical mechanisms and take advantage of different nano-materials including NPs, nanotubes, nanowires, nanochannels, quantum dots, graphene, and graphene oxide have been massively developed and reported (Chamorro-Garcia and Merkoçi, 2016). Particularly, the potential applications of various nanomaterial-based biosensors in medical fields have been deeply explored. For instance, nanobiosensors based on fluorescent NPs, antisense oligonucleotide (ODN)-loaded cyanoacrylate nanospheres, and labeled colloidal NPs are designed and produced for biomedical theranostic, especially for tumor detection and systematic studies on biological processes and cell activities (Brigger et al., 2012; Wu et al., 2020). Due to its wide range of applications, especially in the ultra-sensitive early clinical diagnosis and long-term health monitoring through biomarkers, nanobiosensors have shown a huge impact on traditional medical practices (Attaallah et al., 2020).

On the other hand, though novel and versatile nanobiosensors with great potential in future medical diagnosis and treatment monitoring are continuously designed and processed, their possible biological toxicity and the unknown side effects through complex interactions with biological tissues have set obstacles to their conversion from research and design (R&D) to clinical practice. Meanwhile, along with the rapid development of smart theranostic nanomaterial-based biosensors, emerging the problem of how to improve the positioning and circulation effectiveness of *in vivo* biosensing elements, as the accurate, stable, and long-term perception and transmission of diagnostic/therapeutic signals from targeted sites, as well as the controlled drug delivery to the selected positions are main factors decisively affect the clinical applications of nanobiosensors.

2.2. DDSs and their applications in biosensing

The idea of beneficially influencing the bio-distribution and pharmacokinetics of the administrated drugs and sensing elements has led to the origination of drug delivery systems (DDSs), which include the concepts of temporal (i.e., rate of release) and spatial (i.e., site of release) control of the pharmacokinetic parameters of administrated agents by integrating them with other chemicals, administration devices or processes (Oppenheim, 1981). As early as the 1970s, it has been realized that in addition to the administration, the therapeutic effects and the long-term efficiency of continuous *in vivo* biosensing is determined by the attainment of an active drug and the functioning biosensing element with an appropriate concentration in the target site of the organism (Juliano, 1978). New and emerging DDSs contain oral drug delivery systems, which deliver theranostic agents to specific sites of the gastrointestinal tract, injectable and implantable delivery systems, in which particulate or cellular carriers and chemical or physical releasing approaches are required, and other noninvasive delivery systems, such as transdermal, respiratory, intranasal and ophthalmic delivery systems (Robinson and Mauger, 1991). In the development of nanobiosensors, the excellent design of novel compact nanobiosensors that can simultaneously detect several analytes with low detection limitation, the convenient and low-cost manufacture of nano-biosensing elements without pollution and harmful by-products, as well as the effective and non-toxic *in vivo* delivery of nanoscale devices with sophisticated sensing mechanisms are the key research focuses (Yadav et al., 2011). Nanomaterials endowed with sensing functions have been widely used in drug delivery as "drug reservoirs" to achieve sustained and targeted delivery of theranostic agents with desirable *in vivo* stability, biodistribution, imaging enhancing properties, and drug-releasing mechanisms (Faraji and Wipf, 2009; Jahangirian et al., 2019). One big challenge for long-term *in vivo* applications of engineered nanoprobes and nanosensors is the safe and effective delivery of nano-devices to target positions with long blood residency, which requires delivery methods that are able to avoid their biotoxicity and quick clearance in the circulation. In DDSs, the rapid immune clearance of drug-loaded nanomaterials especially by the mononuclear phagocytic system (MPS) in the liver and spleen is a

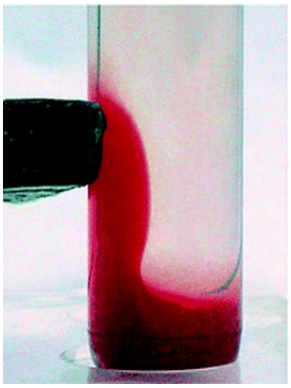
critical obstacle for the *in vivo* applications of nano drugs and nanobiosensors and dramatically limits the effective accumulation in the diseased site (Park, 2014). Meanwhile, the cellular accumulation and clearance of nanomaterials, especially NPs, strongly depend on the particle size and the cell type (Tiwari et al., 2012). Normally, particles of less than 10 nm are rapidly removed through the renal system and extravasation process, and particles over 200 nm are mechanically filtered through the spleen and liver via the reticuloendothelial system (RES) that removes the foreign objects, which is also mediated by opsonization due to surface absorption of proteins and may be simultaneously influenced by temperature (Alexis et al., 2008; Wang et al., 2011). Small (<100 nm) particles were found to have higher skin/tumor accumulation due to lower liver and spleen clearance, and large (>15 μm) particles, which can be fatal according to the dose, are removed from the circulation by mechanical filtration of capillaries (Alexis et al., 2008; Petros and DeSimone, 2010). Among several strategies proposed to decrease MPS uptake, utilizing cells as natural delivery vehicles are actively explored to address the limitations of rapid clearance and poor targeting in DDSs (Batrakova et al., 2011). With the development of more than three decades, cell-based DDSs have become the research focus of modern DDSs attributing to their unique advantages over traditional drug delivery methods, including improved pharmacokinetics (e.g. prolonged *in vivo* circulating lifetime and better bioavailability), inhibited bio-toxicity of novel engineered theranostic agents, and the precisely controllable delivery and release of the agents (Pierigè et al., 2008).

RBCs are the most common and abundant circulating cells with remarkable features that make them inherently biocompatible carriers for intravascular delivery. Therefore, among various cell-based DDSs utilizing nonliving bacterial cells, macrophages, and a large variety of transduced cells, the blood-cell based, especially the RBC-mediated DDSs have the potential to deliver various objects including molecule/protein drugs, bioactive enzymes/peptides, and engineered nano-agents (Sun et al., 2017). RBC-mediated DDSs also possess other superior properties, including the ability to achieve membrane functional modification to enhance the targeted delivery and release. Cinti. et al. have developed an erythro-magneto-HA virosomes DDS by encapsulating fluorescent superparamagnetic NPs and the therapeutic drugs into erythrocytes while attaching Hemagglutinin (HA) viral spike fusion proteins to the erythrocyte membranes to achieve magnetic-directed and cell-fusion induced drug-releasing inside the target cells, which appreciably improved the efficiency and efficacy of cancer therapy (Cinti et al., 2011). Undoubtedly, the RBC-mediated DDSs have greatly promoted the preclinical and clinical development of nano-devices featuring unique advantages in intelligent biosensing for early diagnosis and precision therapy (Jiang et al., 2018). Various approaches for theranostic nano-agents for different clinical application purposes to be incorporated into RBCs or attached on the RBC surfaces before intravascular injection have been investigated and demonstrated to optimize nano-drug-carriers circulation and improve the pharmacokinetics (Tan et al., 2015). Nevertheless, studies have shown that both the characteristic parameters of nano devices (e.g., size, geometry, surface chemistry, and composition) and the interactions of nano devices with biological barriers have direct impacts on their biodistribution and pharmacokinetic properties (Alexis et al., 2008; Petros and DeSimone, 2010; Wang et al., 2011). The adverse consequences of interactions between drug-loaded nano-biosensing elements and biological tissues, especially with carrier RBCs have been reported, as the blood exposure of nano devices is unavoidable in medical research and clinical diagnosis and therapy (Glassman et al., 2020; Wayteck et al., 2016). It has been proved that the integration of diagnostic *in vivo* biosensors and the DDSs for delivery of therapeutic agents enables the construction of theranostic platforms, providing revolutionary breakthroughs to the early diagnosis and treatment of cancers (Shi et al., 2014; Yang et al., 2016). It is even claimed that the majority of *in vivo* nanobiosensors are designed for imaging and drug delivery (Sharifi et al., 2020; Shi et al., 2018).

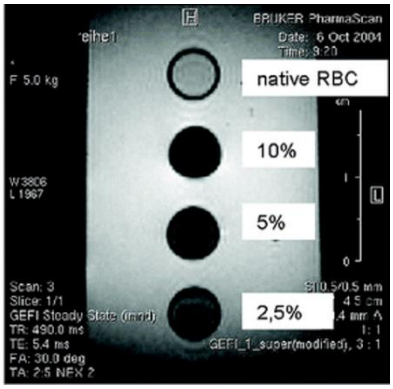
3. RBC-mediated delivery of nano-agents for *in vivo* biosensing

3.1. RBC-mediated delivery of nanobiosensors and nanoprobes for bio-imaging at the single-cell level

Biosensor systems are widely used in multi-modal bio-imaging at the single-cell level including imaging and tracking of single molecules and molecular interactions, visualizing and monitoring specific enzyme activities, and dynamic imaging of bio-fluids for clinical applications (Stawarski et al., 2014; Tran et al., 2018). With the support of nanomaterials, the design of this kind of biosensor imaging system is more diversified and the performance is further promoted. For example, organic fluorophore and chimeric green fluorescent protein have been applied to small-molecule sensing and monitoring in the biological systems including mammalian cells (Rizzuto et al., 1995). Paramagnetic or superparamagnetic NPs are widely used as contrast agents enhancing the sensitivity and specificity of magnetic resonance imaging (MRI) (Brähler et al., 2006). Au-NPs find widespread application in bioimaging as optical markers (Gao et al., 2013). However, fast phagocytosis and resulting short blood pool half-life time of the contrast/imaging agents in bioimaging of tissues and organs are the main factors limiting the clinical application of novel biosensors (Bahmani et al., 2013; Brähler et al., 2006). With cell-assisted methods, especially RBC-mediated DDSs, the improved biocompatibility and *in vivo* stability, especially the prolonged systemic circulation lifetime have expanded the utility of novel nanomaterials in the design of biosensing systems. RBC-based nanoprobes have enabled diagnostic bioimaging with high spatial resolution and distinct curative effects (Bahmani et al., 2013). Based on the RBC membrane-derived vesicles enveloping the triple-doped zinc gallogermanate nanostructures (ZGGO) coated with mesoporous silica, Liu. et al. have synthesized the RBC-mimicking nanocarrier ZGGO@mSiO2 that could bypass phagocytosis by macrophages and systemic clearance by mutual organ filtration with long-term near-infrared luminescence stability, which is suitable for *in vivo* bio-imaging and in situ monitoring of tumor-growth inhibition (Liu et al., 2018). By similar RBC-membrane coating method, Gao. et al. have obtained long-circulating erythrocyte-mimicking Au-NPs that are more effective than free particles in accumulating preferentially at the tumor site through passive or active targeting mechanisms, and enter the cells through endocytosis, providing sensitive and long-term tumor detection and monitoring (Gao et al., 2013). Ahn. et al. extracted the discrete positional information in the blood flows by dynamic X-ray tracing of the surface-modified Au-NPs of around 20 nm that were incorporated into natural RBCs by hypotonic loading with effective image contrast (Ahn et al., 2011). With indocyanine green (ICG) as NIR-transducing exogeneous material encapsulated by red cell membranes, Bahmani. et al. reported the fluorescent imaging of human dermal microvascular endothelial (HDME) cells with the ability to destroy the cells through photothermal effects following laser irradiation at 808 nm (Bahmani et al., 2013). With osmotic swelling method, superparamagnetic iron oxide nanoparticles (SPIONs) with sizes comparable to many macromolecules were loaded and entrapped inside RBCs, circulating together with natural RBCs in the bloodstream and showed extended *in vivo* survival times of maximum 120 days. As shown in Figure 1a, SPIONs-loaded RBCs exhibit magnetic properties and respond to the external magnetic field, which demonstrates a higher contrast than the natural cells alone, even at a low concentration of SPIONs-loaded RBCs as shown in Figure 1b (Brähler et al., 2006). With RBC-assisted method, such contrast agents that can circulate in the bloodstream for a long time are super beneficial to the imaging of the circulatory system, for example in the assessment of the postoperative period in terms of monitoring the process of vascular healing and real-time detection of internal bleeding (Antonelli et al., 2013).



(**a**)



(**b**)

Figure 1. (**a**) Illustration of RBCs loaded with SPIONs by osmotic swelling method responding to a permanent magnet attached to the wall and moved upward slowly. (**b**) MRI of native whole blood and blood samples containing 10%, 5%, and 2.5% SPIONs-loaded RBCs. Reproduced with permission from Brähler, M. et al., Nano Lett; published by American Chemical Society, 2006.

3.2. RBC-mediated delivery of nanobiosensors as biomarkers for diagnostics

Biosensors including electrochemical and optical nanosensors that could serve as biomarkers for clinical diagnosis and disease screening have been receiving great attention for their diagnostic potential in biomedical assessments such as rapid and accurate detection of various autoimmune diseases, infectious diseases, and early-stage cancers (Ghorbani et al., 2019; Sharifi et al., 2019; Sin et al., 2014). Particularly, significant differences between cancerous and normal cells in intracellular and extracellular environments, and the special characteristics of tumors (e.g., neovasculature, pH, and physicochemical changes) have motivated the development in the realm of site-specific delivery and detection of novel diagnostic sensors based on nanomaterials and different stimulus-responsive mechanisms (Portney and Ozkan, 2006; Salvati et al., 2015). Novel electrochemical biosensors with enhanced sensitivity and outstanding stability based on nanomaterials including functionalized gold nanorods combined with graphene oxide and nitrogen-doped graphene (NFG)/Ag-NPs/Polyaniline (PANI) three-layer nanocomposite were built to selectively detect the circulating miRNA-155 or tumor cells in the blood, the level of which elevated through the interconnection with breast cancer (Azimzadeh et al., 2016; Salahandish et al., 2018). Combining the morphological changes of RBCs under the different PH of the blood and the optical tweezers (OTs) technique, Li. et al. recently proposed a living biosensor based on an *in vivo* RBC waveguide controlled and monitored by the optical gradient force formed by the strong penetrating infrared laser light (980 nm) emitted from the two ends of tapered fiber probes (Li et al., 2019). In recent years, superparamagnetic iron oxide (SPIO) NPs have been extensively used as clinical MRI contrast agent, despite the rapid clearance by the Kupffer cells in the spleen, liver, and bone marrow, SPIO-NPs have improved the detection rate of tumors in these tissues (Antonelli and Magnani, 2014). Meanwhile, superparamagnetic NP conjugates that reversibly self-assemble into stable nanoclusters in aqueous solution under the excitation of a corresponding target (e.g., the formation of serotonin attached-iron oxide NP clusters in the presence of myeloperoxidase that is associated with inflammation and atherosclerosis), have been utilized as sensitive magnetic nanosensors to detect the peroxidase activity and amplify the MR signal in the diagnosis of peroxidase-induced diseases (Perez et al., 2004, 2002). By encapsulating into RBCs, the applications of long-circulating MRI contrast agents have been extended to the investigation of vascular system and diagnosis of cardiovascular diseases (Antonelli et al., 2017; Antonelli and Magnani, 2014). Furthermore, the combination of nanobiosensors and therapeutic agents allows simultaneous diagnosis and disease treatment (Li et al., 2016). By applying optical torque on the trapped RBCs, the polymer microparticles serving as drug carriers could be directed to the target region by the driving flow generated by the RBC micromotor, which realizes the diagnosis and treatment of PH-related diseases on a single platform. Predictably, supported by the RBC-based DDSs, the improved biocompatibility and effective targeted delivery of the diagnostic biosensing elements will further stimulate the applications of selective *in vivo* imaging, promote the development of portable and easy-to-use point-of-care diagnosis, increase the precision of clinical tests, and improve the therapeutic efficacies (Ahmed et al., 2014).

3.3. RBC-mediated delivery of nanobiosensors for biosensing of biomolecules and cellular activities

Nanobiosensors and nanoprobes are revolutionizing devices for in-situ sensing of biomolecules in individual living objects. The analytical analysis of cellular activities and precise detection of particular chemical species in target locations within a single cell have greatly improved our understanding of cellular functionalities and fundamental biological processes, and stimulated cell biology research to achieve revolutionary breakthrough (Halstead et al., 2015; Li et al., 2018; Vo-Dinh et al., 2006). For instance, the integration of nano-transducers with fluorescent sensing techniques, especially the incorporation of such fluorescent nanomaterials with biological sensing and delivery

elements, has made it possible for convenient real-time glucose monitoring (Chen et al., 2018; Thomas et al., 2016). Detection of various biomolecules like proteins, growth factors, and nucleic acids by monitoring changes in an electrical signal by different field-effect transistor biosensors has become possible by utilizing graphene-based electrochemical biosensors (Kumar et al., 2015; Lu et al., 2009; Shao et al., 2010). Plasmonic noble metal nanomaterials (e.g. Au, Ag, and Au@Ag core-shell nanocubes) have been applied in localized surface plasmon resonance (LSPR) sensing of molecular activities (Maxwell et al., 2002; Zhang et al., 2018). The finely tuned LSPR properties of Au-NPs during the formation and disassembly of aggregates of surface-modified or unmodified Au-NPs in the act of specific molecule targets, including proteins, ions, small molecules, and DNA, have found great potential in selective cellular analysis and molecule detection in diluted whole blood (Wang et al., 2009, 2008). Particularly, cell-based detection and delivery systems have become promising tools for the rapid bio-sensing of single molecules and analytical measurements of cellular activities (Banerjee et al., 2008). For instance, based on semiconductor NP-based fluorescent biosensing system combined with metalloprotein design techniques for selective binding, spatial and time-dependent variations of exchangeable Pb^{2+} ion concentrations within and around RBCs, which is closely related to the intelligence quotient (IQ)-level, blood pressure, and motor responses of children, were detected after the incorporation of the designed nano-sensors into RBCs by reversible membrane pore-opening/resealing method (Shete and Benson, 2009). By immobilizing on RBCs, gold-coated Fe_3O_4 core-shell nanocomposite was used as a cellular biosensor for hydrogen peroxide detection through RBCs-Fe_3O_4/Au interface (Chen et al., 2010).

4. Mechanisms of RBC-mediated delivery of nanobiosensors

4.1. Principles of cargo loading onto RBCs

Various procedures have been proposed to deliver and transport therapeutic and diagnostic nano-agents through human RBCs so that they can circulate with natural RBCs in the bloodstream for a long time, reach the target position, and be released and accumulated in certain tissues or organs to achieve excellent therapeutic or diagnostic performances (Hu et al., 2012). Numerous therapeutic agents including proteins, nucleic acid, viral agents, and novel nano-drugs have been explored to be carried and delivered through "carrier RBCs" by various loading techniques, including covalent and physical interactions with red cell membrane, drug encapsulation into RBCs, and RBC membrane-derived vesicles (Magnani et al., 2002; Sun et al., 2017; Zarrin et al., 2014). One kind of the RBC-based DDSs is achieved by passive NP-delivery on the RBC membrane surface and is typically referred to as RBC-hitchhiking. It is widely used due to its easy preparation, excellent performance in increasing the circulation lifetime of NPs in the bloodstream, and can achieve *in vivo* targeted delivery. The mechanism is the direct interaction between NPs and the exterior RBC membrane surface during the *in vitro* incubation and pretreatment before injection through physical adsorption onto the membrane, which is mainly based on non-covalent interactions such as ion exchanging, ion pairing, hydrogen bonding, non-site specific van der Waals and hydrophobic forces (Heinz et al., 2017; Yoon et al., 1998). Therefore, the strength, stability, and distribution of surface adsorption of NPs on the RBC membrane as illustrated in Figure 2 (Avsievich et al., 2019), are dependent on the particle charges, sizes, and colloidal stability (Zelepukin et al., 2019). Since RBCs are negatively charged, the surface charge (zeta potential) of the NPs has effects on both the adsorption rate of NPs to RBCs and the circulation lifetime of NPs in the vascular system. For example, the immunoglobulin G (IgG) coated lysozyme-dextran nano-gel (IgG-LDNG, with a zeta potential of 0.43+/-0.16 mV) has nearly four times the adsorption efficiency of the non-coated LDNG counterpart (with a zeta potential of -0.42+/-0.09 mV) (Pan et al., 2018). A study investigating RBC-hitchhiking of small sub-200 nm NPs also showed that the circulation prolongation was only observed for positively charged NPs attributed to strong electrostatic interactions between NPs and negatively charged RBCs (Zelepukin et al., 2019). Other advanced cargo-loading methods relying on RBC membrane-binding via covalent connection through chemical linkers include inserting lipid section of molecules into the lipid bilayers of RBC membrane, constructing biotin-avidin-bridges through lipid-anchors or chemical modification,

triggering coupling reaction mediated by the 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and stabilized with N-hydroxysuccinimide (NHS), and inducing antibody-membrane receptor binding via antibody-drug conjugates, have been systematically summarized in several reviews and illustrated in Figure 3 (Moghimi and Szebeni, 2003; Muzykantov, 2010; Sun et al., 2019; Yan et al., 2017).

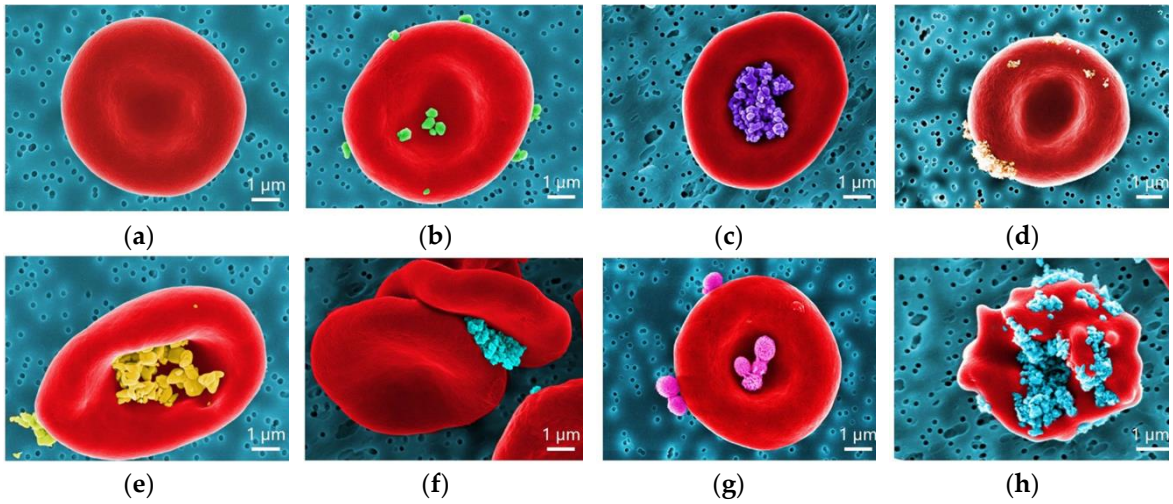


Figure 2. Fake color SEM images of (**a**) a normal human RBC and the non-covalent attachment and localization on the RBC membrane surface of different inorganic NPs including: (**b**) TiO_2 NPs of 250 nm; (**c**) TiO_2 NPs of 180 nm; (**d**) TiO_2 NPs of 15 nm; (**e**) ZnO NPs of 270 nm; (**f**) nanodiamonds of 100 nm; and (**g**) polymeric NP with size of 600 nm; (**h**) observed formation of RBC echinocyte induced by a large amount of surface adhesion of nano-diamonds. Reproduced with permission from Avsievich, T. et al., Sci Rep; published by Springer Nature, 2019.

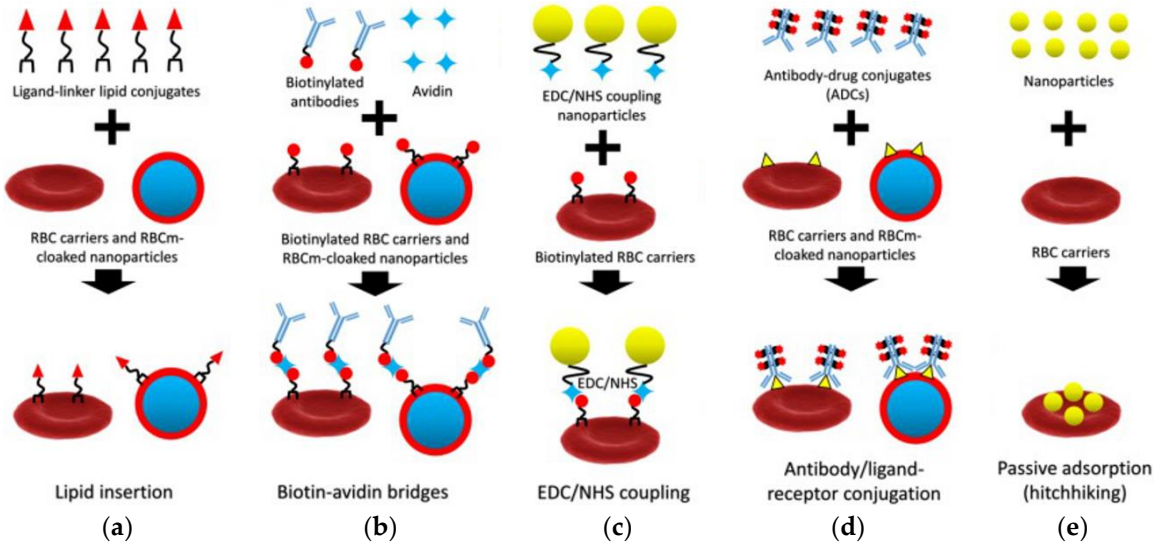


Figure 3. Illustration of several advanced drug loading techniques based on RBC-membrane or outer-surface of RBC membrane-derived vesicles by covalent and non-covalent interactions including (**a**) lipid insertion, (**b**) biotin-avidin bridges, (**c**) EDC/NHS coupling, (**d**) antibody/ligand-receptor conjugation, and (**e**) RBC-hitchhiking. Reproduced with permission from Sun, D. et al., Theranostics; published by Ivyspring International Publisher, 2019.

In contrast to NP delivery and transport based on the cell surface, drug-loaded NPs can be impregnated into RBCs while maintaining the integrity of the cell membrane, with RBCs serving as natural compartments protecting encapsulated cargos. Utilizing the semi-permeability of the RBC membrane, this method provides ideal bio-reactors to isolate and retain protein drugs while allowing enzyme substrates to enter to achieve controlled activating of inactive prodrugs into active drugs

through enzymatic activities (Magnani et al., 2002). In essence, most theranostic agents that are non-toxic to RBCs including novel nanomedicines, peptides, and nucleic acids can be encapsulated into RBCs without damage. One universally used procedure is based on the reversible opening of the pores on the RBC membrane that are large enough (ranging from 10 to 500 nm) to permit the crossing of external agents under low osmotic pressure as shown in Figure 4 (Jeewantha and Slivkin, 2018). This process is able to entrap a large variety of drugs into RBCs uniformly, resulting in final products with good reproducibility, reliability, and viability, thus has been developed into the industrial scale and is widely selected by experts and researchers (Moghimi et al., 2001). With this method, highly fluorescent and photostable SiNPs loaded with chemotherapeutic drug doxorubicin (DOX) molecules (SiNPs-DOX complex) were incubated with RBCs in hypotonic buffer and successful encapsulated into RBCs (SiNPs-DOX@RBCs) in subsequently reconstituted isotonicity (Jiang et al., 2018). The ultraviolet and visible (UV-vis) absorption spectra of SiNPs-DOX@RBCs confirmed characteristic photoluminescence (PL) peaks of both SiNPs and DOX, and the confocal laser scanning microscopy (CLSM) images indicated co-localization of SiNPs and DOX. The SiNPs-DOX encapsulated in RBCs had a longer blood circulation time, whereas their intracellular distribution and cancer cell killing effectiveness were not modified, indicating RBCs as outstanding drug carriers.

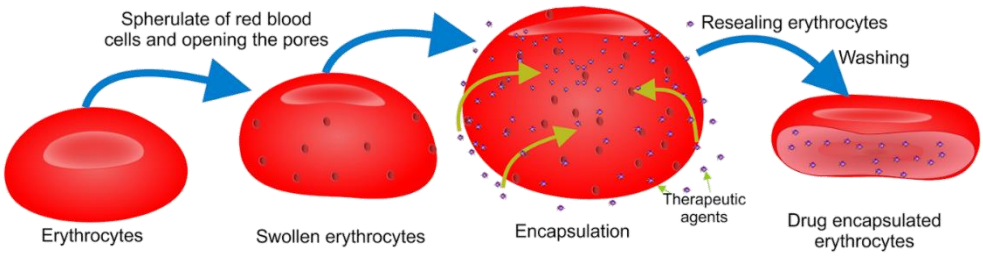


Figure 4. The encapsulation of nano-agents within human RBCs by loading external nano-agents through pores on the RBC membrane opened in a hypotonic condition and resealed in the restored osmolarity followed by washing out the un-loaded substances. Reproduced with permission from Jeewantha, H.M.A. and Slivkin, A.I., Russ Open Med J; published by Limited liability company «Science and Innovations» (Saratov, Russia), 2018.

Furthermore, the advances in molecular biology and nanotechnology have spurred the design and synthesis of man-made vehicles that can mimic the remarkable mechanobiological and chemical-biological properties of natural RBCs, which further inspired the development of the DDSs utilizing advantageous features of RBCs based on the biomimetic strategies of nano-material-cores coated with red cell membranes (Hu et al., 2012).

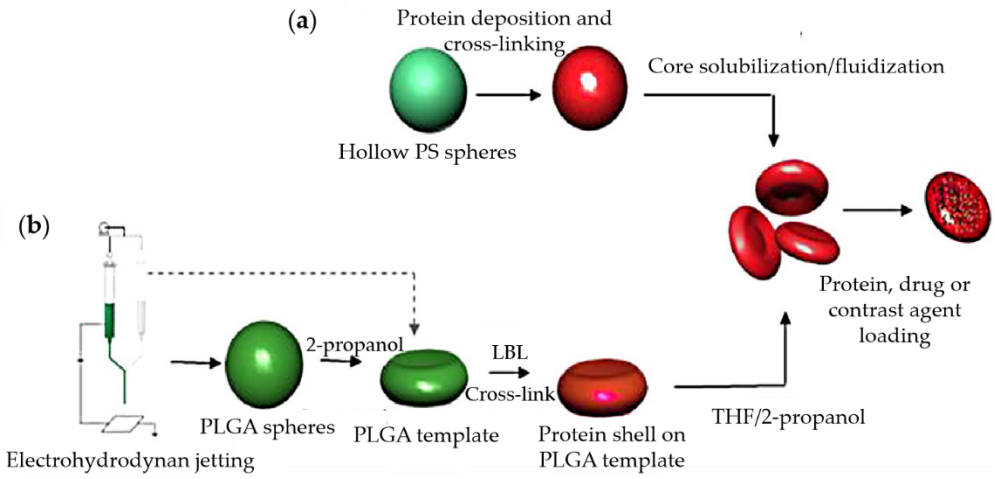


Figure 5. Demonstration of the synthesis of RBC-mimicking particles by (**a**) solvent or heat-induced fluidization of hollow polystyrene (PS) particles that results in biconcave RBC-shape, and by (**b**) RBC-shaped poly (lactic-co-glycolic acid) (PLGA) particles produced by electrohydrodynamic jetting in 2-propanol. Cationic and anionic polymer and protein shell on the outer surface were deposited by LBL

method. Reproduced with permission from Doshi, N. et al., Proc Natl Acad Sci; published by National Academy of Sciences, 2009.

The preparation and characterization of anti-tumor erythrocyte-mimicking NPs, the future potential regarding functional modulation of RBC membrane, and fusion of various cell membrane surface properties in the design of versatile coating membranes are properly summarized in a recent review (Xia et al., 2019). Particularly, synthetic carriers that can mimic the shape, mechanical, electrical, and biological properties of RBCs while possessing special engineering qualities are designed in the fabrication of RBC-like particles, which could fill the gap between synthetic materials and natural organisms (Pacheco-Jerez and Jurado-Sánchez, 2019). Figure 5 demonstrated a RBC-mimicking particle synthesis technique based on hollow polystyrene (PS) particle that has good elasticity (Doshi et al., 2009). Morphological modification is based on solvent or heat-induced fluidization that induces shape collapse, and cationic and anionic polymer deposition on the particle surface can be achieved by a layer-by-layer (LBL) self-assembly method (Kurapati et al., 2019). In conclusion, RBC-based drug delivery (loading) methods are summarized in Table 1.

Table 1. *Cargo-loading methods used in RBC-mediated DDSs.*

Method	Loading drug	Mechanism	References
Direct coupling with RBC membrane surface	Biotinylated NPs, antibodies, and streptavidin.	Biotin-avidin bridges and lipid insertion.	(Cheng et al., 2010; Muzykantov et al., 1996; Nobiron, 2003; Zaltzman et al., 1995)
	A large variety of inorganic NPs including magnetic NPs.	Indirect conjugation of NPs and RBC-membrane through direct EDC/NHS coupling based on amide bonds formation.	(Sun et al., 2019)
	Therapeutic drugs that can be conjugated with antibodies, antibody fragments, peptides or other ligands that can bind to the RBCs membrane.	Antibody/ligand-receptor conjugation	(Chen and Li, 2020; Muzykantov et al., 1985)
Physical adsorption onto the RBC membrane surface	Theranostic NPs, such as cancer drug DOX, positively charged chitosan-coated NPs (CTSs).	Ion exchanging; ion pairing; hydrogen bonding; non-site-specific van der Waals and hydrophobic forces.	(Glassman et al., 2020; Heinz et al., 2017; Yoon et al., 1998)
Encapsulation into natural RBCs	Peptide-like drug (e.g. enalaprilat, anti-HIV peptides), enzymes, and antibiotics.	Osmosis-based methods including hypotonic hemolysis, dilution, dialysis, pre-swelling, and osmotic pulse.	(Gutiérrez Millán et al., 2004; Hamidi and Tajerzadeh, 2003; Tajerzadeh and Hamidi, 2000)
	Large particles such as virus (up to 100 nm in diameter), enzyme and small molecules.	RBC endocytosis through membrane-active drugs induced membrane-lined vacuoles.	(Ben-Bassat et al., 1972; Patel et al., 2009)
	Enzymes such as alcohol dehydrogenase (ADH) and/or acetaldehyde dehydrogenase (ALDH)	Electrical pulse method based on electrical shock induced irreversible permeability changes to RBC membrane.	(Lizano et al., 1998)
	Membrane-impermeable solutes (e.g. inositol hexaphosphate (IHP))	Fusing/merging of the outer lipid bilayers of small unilamellar lipid vesicle with RBC membrane in contact.	(Nicolau and Gersonde, 1979)
Man-made vehicles mimicking natural RBCs	Proteins, nano-drugs (e.g. iron oxide nanoparticles), contrast agents, etc.	Inducing morphological and mechanical modification to spherical polymeric particles to mimic the key structural attributes of RBCs.	(Doshi et al., 2009)
RBC membrane-derived vesicles	A large variety of particle substrates, such as polymeric NPs ranging from 65 to 340 nm in diameter.	Coating NPs with natural RBC membranes (e.g. by mechanical force exerted in extrusion of the membranes-NPs mixture through a 100 nm polycarbonate porous membrane) isolated from whole blood (e.g. by hypotonic hemolysis).	(Hu et al., 2011; Luk et al., 2014; Yan et al., 2017)

RBC-NP interaction also exists in free NP *in vivo* delivery in a vascular system as illustrated in Figure 6, which is one of the main determinants of the NP dispersion rate, particle motion, and targeted binding due to the Newtonian flow properties and particle nature of blood. The simulated results on the diffusion and adhesion kinetics of NPs in the complex vascular environment based on the Immersed Finite Element Method (IFEM) platform provides a new model for understanding the dynamic transport and targeted delivery of drug-loaded NPs (Tan et al., 2012).

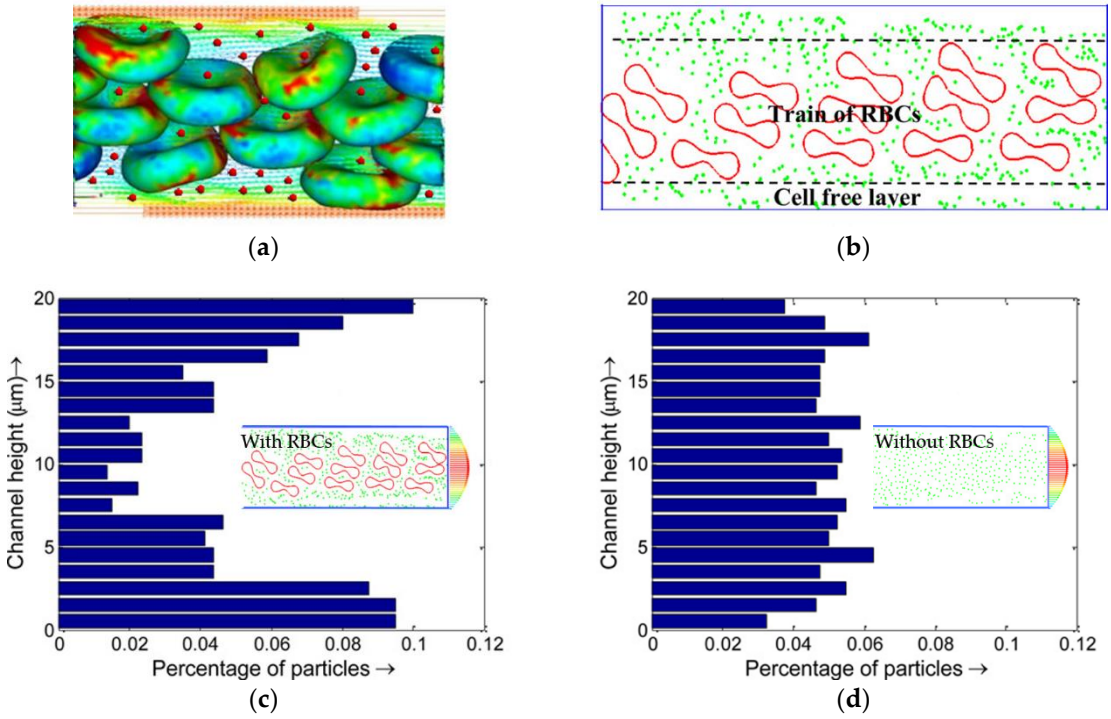


Figure 6. (**a**) 3D modeling of RBCs as soft thin membranes enveloping cytoplasm that has the same viscosity as the extracellular plasma and the NP–RBC interaction within blood vessels; (**b**) Cross-section of RBC-NP interaction and the illustration of a depletion layer (cell free layer) near the vessel wall; (**c**) Simulated result of NP-distribution in blood vessel with RBCs and (**d**) without RBCs at time t= 7.5 s. Reproduced with permission from Tan, J. et al., Soft Matter; published by Royal Society of Chemistry, 2012.

4.2. Principles of cargo releasing from RBCs

One of the drug-releasing mechanisms is to promote the spontaneous binding with autologous immunoglobulin by modifying the circulating cargo-loaded erythrocytes, resulting in the macrophages recognition and phagocytosis of the opsonized RBCs to enhance the release of the carrying cargo within macrophages (Magnani et al., 2002). Another way for the encapsulated drugs to flow out of the carrier RBCs is through membrane diffusion, the rate of which is dependent on the speed at which a molecule passing through a lipid bilayer of the cell membrane (Patel et al., 2009). For particular agents carried on the RBC surface, the main mechanism for particle detachment is mechanical scraping of the NPs from the carrier cells by the shear forces applied by the surface of capillaries, especially when cargo-loaded RBCs squeeze through small capillaries, as demonstrated in Figure 7 (Anselmo et al., 2013; Brenner et al., 2018; Zelepukin et al., 2019). Additionally, various physical, chemical, and biological stimuli-responsive releasing mechanisms, including thermo-responsive, magnetic-responsive, electrical-responsive, light-responsive, mechanical-responsive, ultrasound-responsive, pH-responsive, biomolecular-responsive, etc., have been thoroughly studied for the development of site-specific and drug-release-time controllable smart DDSs (Goulet-Hanssens et al., 2016; Karimi et al., 2016). As an example of light-induced thermo-responsive drug-releasing, when particles with high photothermal conversion efficiency are encapsulated in carrier RBCs together with the desired nano-drugs, for instance, by incorporating albumin-bound near-infrared

(NIR) dye and DOX within the RBC platform, the drug-releasing can be triggered remotely by destroying the red cell membrane via light-induced heating effect (Sun et al., 2015).

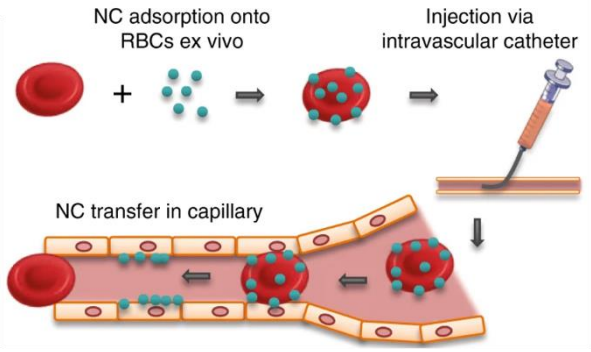


Figure 7. Schematic of the mechanical scraping of nanocarriers (NC), which were adsorbed on the RBC surface and injected into the circulation system through intravascular injection, by the shear stress applied by a small capillary. Reproduced with permission from Brenner, J.S. et al., Nat Commun; published by Springer Nature, 2018.

5. Advantages and challenges of RBC-mediated delivery of biosensing nano-agents

5.1. Advantages of RBC-mediated delivery of biosensing nano-agents

Circulation prolongation is one of the main advantages of RBC-delivered biosensing nano-agents. For intravenous injection of free NPs, the particle uptake in liver and spleen is the most significant and shows particle size-dependent organ distribution. Among different sized (10-250 nm) spherical-shaped gold NPs (Au-NPs), large NPs mainly distributed in blood, liver, and spleen, whereas smaller (10 nm) Au-NPs were found in various organs including blood, liver, spleen, kidney, testis, heart, lung and brain, and have the most widespread organ distribution in rat at 24 h after intravenous injection (De Jong et al., 2008). Compared with freely administered NPs, the blood level over 24 h and the accumulation in the lungs of the spherical polystyrene NPs carried and transferred by RBCs through non-covalent attachment to the RBCs surface via electrostatic and hydrophobic interactions were increased by ~3-fold and ~7-fold respectively, and the lung targeting was further improved with antibody attachment to the NPs (Anselmo et al., 2013). Theoretically, the number of NPs in circulation is related to the number of carrier RBCs and the number of NPs carried by each carrier RBCs as follows (Chambers and Mitragotri, 2004):

$$\mathrm{N} = \sum_{m=1}^{\infty} \mathrm{m}\, N_m \,, \tag{1}$$

where N is the total number of NPs in the circulation, which can be estimated by flow cytometry methods when the number (N_m) of RBCs carrying m particles can be tracked. With the 450 nm diameter polystyrene NPs, a model of NP clearance as described by Equations (2-4) according to NP loading number on the RBCs has been established and verified experimentally (Chambers and Mitragotri, 2004; Stoltze, 2000):

$$X_1 = \frac{N'_1}{N_1} = \left(1 + \beta kt + \frac{1}{2}\gamma k^2 t^2\right) e^{-kt} = (1 + \frac{N_1+N_2}{N_0} kt + \frac{1}{2}\frac{N_1+N_3}{N_0} t^2) e^{-kt} \,, \tag{2}$$

$$X_2 = \frac{N'_2}{N_2} = (1+\propto kt) e^{-kt} = (1 + \frac{N_2+N_3}{N_0} kt) e^{-kt} \,, \tag{3}$$

$$X_3 = \frac{N'_3}{N_3} = e^{-kt} \,, \tag{4}$$

where N_m (m=0, 1, 2, 3) is defined in Equation (1) as the number of initial RBCs not carrying NPs (m=0) and carrying only one (m=1), two (m=2), or three (m=3) NPs on the membrane surface and N'_m (m=1, 2, 3) refers to the number of RBCs carrying m NPs that are still in the circulation after the time t. The ratios α, β, and γ can be measured experimentally and the clearance coefficient k can be obtained by fitting the experimental curve to the theoretical model. As shown in Figure 8, the removal of NPs from the surface of carrier RBCs follows the first-order kinetics, and higher loading rates result in

faster removal (Chambers and Mitragotri, 2004). Fundamental theoretical models for the simulation of the Brownian adhesion and dispersion of NPs in the circulation, as well as the NP-cell interaction have been established (Tan et al., 2012).

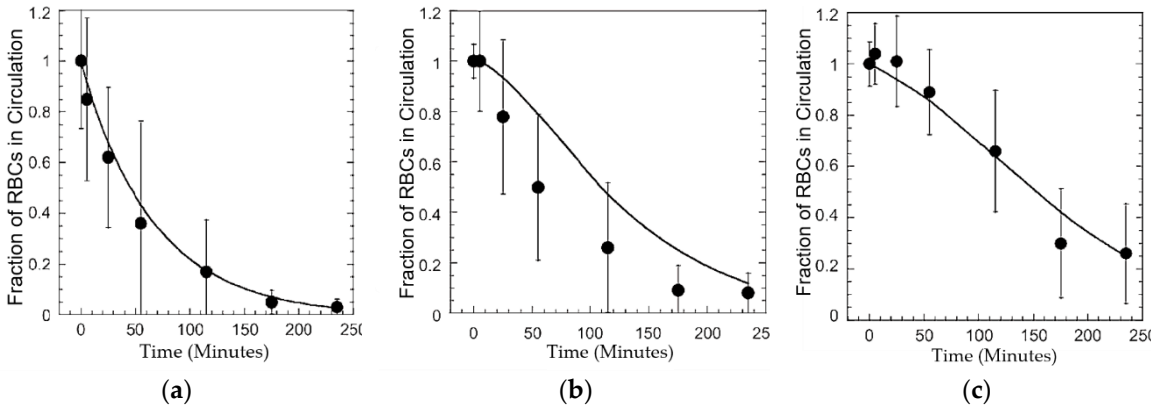


Figure 8. Experimental observation of the fraction of RBCs carrying m NPs (X_m) in the circulation after different time periods when (**a**) m=3, (**b**) m=2, and (**c**) m=1. Black dots are experimental data with deviation and solid lines are theoretical prediction according to Equations (2-4). Reproduced with permission from Chambers, E. and Mitragotri, S., J Control Release; published by Elsevier, 2004.

On the other hand, RBC-delivered small NPs (sub-200 nm range) whose blood circulation lifetime has not been prolonged are still able to achieve high lung accumulation efficiency while the liver uptake is suppressed, indicating that the reversible complexation of NPs with RBCs rather than the blood circulation time is the key factor affecting the efficiency of RBC-delivered NPs. (Zelepukin et al., 2019). Another advantageous property of RBC-based DDSs is that the function of converting inactive prodrugs into active drugs can be achieved through various RBC enzymatic activities. Therefore, a number of prodrugs can be designed and synthesized to be non-diffusible and non-toxic when loaded into RBCs, and will only be released through the red cell membrane to the circulation or specific sites after certain enzymatic hydrolysis within RBCs to achieve targeted delivery (Magnani et al., 2002). Furthermore, the recent success of treatment of melanoma pulmonary metastases by RBC-delivered DOX loaded chitosan (CTS) NPs revealed the therapeutic potential of RBC-hitchhiking of nano-agents in the treatment of lung diseases, especially in the conditions of aggressive small-cell cancer or metastases when the treatment by traditional surgery is hard to realize (Zelepukin et al., 2019). In particular, in such applications where the NPs act as drug carriers, the most suitable nanoparticle carrier can be designed and selected according to the target sites, drug properties, and delivery efficiency, etc., ensuring great improvement possibilities for the RBC-based DDSs. Through RBC-hitchhiking, it is reported that the delivery efficacy and targeted transportation to chosen tissue especially to the first organ downstream of the intravascular injection of a variety of nanocarriers were greatly improved via selective placement of the intravascular catheters, indicating RBC-hitchhiking an effective and clinically applicable drug-delivery platform for the treatment of acute and severe lung, brain, and cardiovascular diseases (Brenner et al., 2018).

5.2. Challenges/problems of RBC-mediated delivery of biosensing nano-agents

As the intravenous administration of cargo-loaded RBCs to the circulating system is mandatory, the feasibility and safety of RBC-based DDSs especially for new clinical applications where nanoparticles, proteins, and genes are theranostic agents have arisen researchers' attention and preliminary evaluations have been made. A detailed conclusion of the mechanism and severe adverse effects of different nanomaterials on erythrocytes, leukocytes, and thrombocytes according to their crucial role in maintaining the well-functioning of the microcirculation within vasculature can be found from a recent review focused on the interaction of NPs with blood elements from a cellular view (de la Harpe et al., 2019). Specifically, a study has shown that polymeric NPs have great biocompatibility as their incubation with both RBCs and large Human Mesenchymal Stem Cells (HMSCS) with a size of 20-30 μm has neither affected the cell-to-cell interaction mechanism nor

caused changes in cell morphology (Avsievich et al., 2020a). Meanwhile, the coupling of spherical polystyrene NPs (PSNPs) to RBC surface did not adversely affect RBC functioning as the biodistribution and organ retention of RBCs were not modified, though the NP carriage at high dose (100:1) decreased the circulation time of the carrier RBCs due to the accelerated removal of RBCs carrying multiple NPs compared to native RBCs (Anselmo et al., 2013). However, the intrinsic hemodynamic characteristics of RBCs are affected by the surface adhesion of NPs. The RBC stiffness, degree of spontaneous aggregation, and hemolysis under shear/oxidative stress were enhanced especially by the adsorption of PSNPs in comparison with LDNG/IgG-LDNG (Pan et al., 2018). Based on the modifications to the main spectral bands of the electronic absorption and vibrational Raman spectra of animal blood hemolysates (horse and dog) after incubation with magnetic NPs, the intensification of hemolysis following the addition of NPs and the complex interactions between colloidal magnetite and the heme group, which involves the iron ions within the hemoglobin, was revealed by the elevated level of hemoglobin, significant changes in electronic transitions, and diminished characteristic peaks in Raman spectra (Creangă et al., 2009). Moreover, silver NPs (Ag-NPs) were found to possess prothrombotic effects on RBCs by enhancing the thrombin generation and the adhesion of RBCs to endothelial cells and to potentiate the procoagulant effects of certain drugs used in chemotherapy. Specifically, RBC procoagulant activity is promoted by phosphatidylserine (PS) externalization and micro-vesicles (MV) generation that can be caused by Ag-NP induced generation of reactive oxygen species (ROS) and related intracellular activity, oxidative stress, and the loss of phospholipid asymmetry due to RBC morphological changes (e.g., echinocytes) as demonstrated in Figure 9 (Bian et al., 2019). Such studies indicate additional risks of Ag-NPs on the population sensitive to thrombosis, including cancer patients.

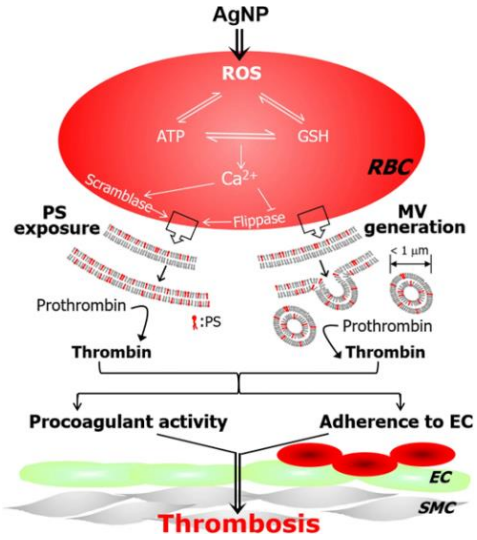


Figure 9. Suggested mechanism for the prothrombotic effects of Ag-NP on RBCs. The Ag-NP induced oxidative stress, intracellular calcium elevation, and adenosine triphosphate (ATP) depleting facilitate coagulation cascade and clot formation in venous thrombosis. Reproduced with permission from Bian, Y. et al., Part Fibre Toxicol; published by Springer Nature, 2019.

In addition to cell viability and hemolysis assays, NP-induced RBC aggregation has been more and more included in hemocompatibility tests of NPs (de la Harpe et al., 2019). Recently, along with the development and improvement of the OTs technique, which is an innovation in laser physics that has been awarded the 2018 Nobel Prize in Physics, detailed rheological properties of blood elements as well as dynamic responses of RBCs to intracellular and extracellular environmental changes have been revealed from a single-cell level (Zhu et al., 2020a). Previous works from our group have thoroughly explored the mutual interplay of blood cells influenced by the intercellular interaction time (Zhu et al., 2019b), dextran solutions with different molecular weight (Avsievich et al., 2018), external He–Ne laser irradiation (Zhu et al., 2019a), and the presence of different NPs (Avsievich et al., 2019). As a consequence, OTs are extremely useful in probing the intercellular activities of

individual cells and hence have the potential to directly evaluate the biocompatibility of novel NPs through their impacts on the cells and cellular activities (Avsievich et al., 2020b; Zhu et al., 2020b). Based on the direct measurement of cell adhesion after incubation with different inorganic and polymeric NPs by OTs, it is reported that compared to polymeric NPs that do not change RBC interaction mechanism, inorganic NPs, especially nanodiamonds, activate cellular interactions and result in enhanced RBC autonomous aggregation, which was confirmed by light microscopy observation as shown in Figure 10 (Avsievich et al., 2019).

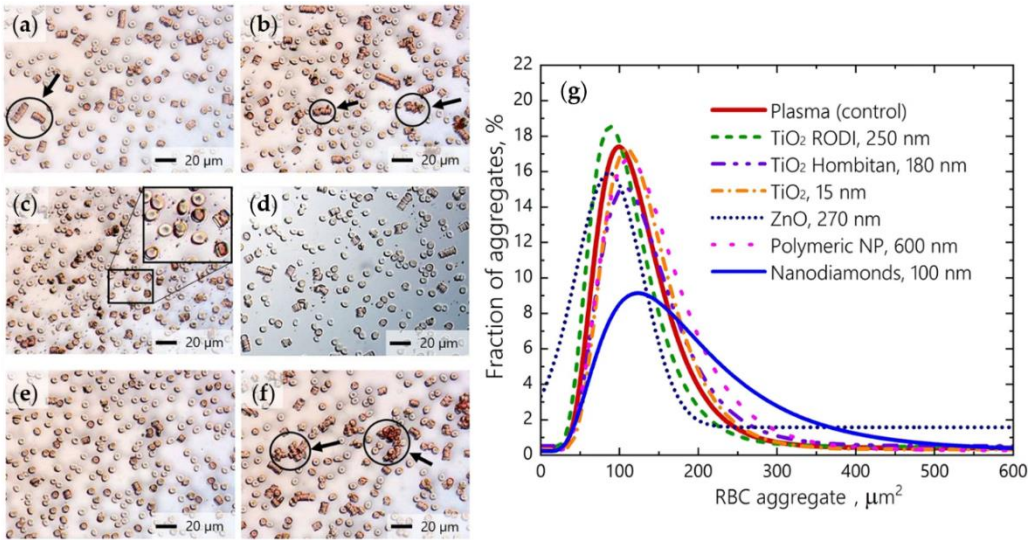


Figure 10. Light microscopy observation of the formation of irregular RBC aggregates (rouleaux) with varied size and shape in the presence of different NPs including (**a**) polymeric NP with size of 600 nm; (**b**) TiO_2 NPs of 250 nm; (**c**) TiO_2 NPs of 180 nm; (**d**) TiO_2 NPs of 15 nm; (**e**) ZnO NPs of 270 nm; and (**f**) nanodiamonds of 100 nm. (**g**) Size distribution of RBC aggregates calculated from images in (**a-f**). Reproduced with permission from Avsievich, T. et al., Sci Rep; published by Springer Nature, 2019.

6. Conclusions and perspectives

In conclusion, with the rapid development in the emerging field of nanomedicine, novel NPs have been investigated in preclinical studies and are being transformed to a great variety of clinical applications including cancer imaging (e.g., with silica NPs) and thermal ablation of tumors (e.g., with gold and iron-oxide NPs). Currently, transport and delivery of NP-based theranostic agents via their covalent or physical interactions with RBCs have been developed rapidly and are currently in different stages of transforming from research and design to pre-clinical and clinical investigations (Sun et al., 2019). The industry of RBC-mediated delivery of theranostic agents consists of more than five major companies/organizations that differentiate themselves by the method adopted to associate drugs with RBCs. ERYTECH (founded in Lyon, France in 2004) and EryDel (founded in Italy in 2007) are two clinical-stage biopharmaceutical companies. ERYTECH has been developing drug-encapsulated RBCs (e.g., L-asparaginase encapsulated donor-derived RBCs) for targeted treatment of rare forms of cancer and orphan diseases using the ERYCAPS technical platform based on the reversible hypotonic and hypertonic osmotic stress. EryDel is specialized in the development of drugs and diagnostics delivery through RBCs via a proprietary medical device technology called Red Cell Loader (RCL), which is capable of non-invasive blood processing at the point of care based on the osmotic pressure for the encapsulation of small molecules, proteins, and diagnostics. Biconcavity (founded in Lilburn, United States in 2017) is committed to facilitating the future of autoimmune therapeutics by developing drug-linked-RBC therapies using the APEX (Activated Protein-Erythrocyte Cross-linking) Method to link peptides/proteins to RBCs. Instead of taking advantage of existing RBCs, Rubius Therapeutics (founded in Cambridge, United Kingdom in 2013) utilizes hematopoietic precursor cells and gene engineering technology to generate RBCs with biotherapeutic protein(s) and pioneered a new era of cellular medicine called Red Cell Therapeutics.

As one of the main concerns of the RBC-mediated DDSs, the evaluation of pathophysiological effects associated with the *in vivo* behavior of nanomaterials, including their interaction with biological objects, such as blood components and human tissue/organs, must be refined and developed to provide sufficient guidance in the safe use and production of nano drugs (Septiadi et al., 2018). Meanwhile, future development requires reliable and non-invasive manipulation of RBCs, especially in the development of more effective drug-loading/encapsulating methods, in the investigation of the underlying mechanism of drug-loading/releasing to achieve reliably controllable targeted delivery, as well as in the case-specific evaluation of biocompatibility of engineered NP-based theranostic agents. Therefore, single-cell techniques, especially OTs that have already shown great potential in studies of RBCs and RBC-NP interactions, will be further improved and applied in the development of RBC-based DDSs (Hu et al., 2012; Zhu et al., 2020a). As the new delivery paradigm based on natural RBCs enables the use of a large variety of novel engineered NP-based theranostic agents with minimum toxicity and promising efficacy, the manufacturing process needs to be further industrialized with improved stability to accelerate the transformation of the RBC-based DDSs to clinical applications (Godfrin et al., 2012; Magnani, 2012). Predictably, based on the advanced technology for optimized nanomaterial design and processing according to special requirements, together with the in-depth investigation of the precise and effective control of drug delivery and releasing via RBC-mediated DDSs, clinically applicable platforms for biosensing applications will be further developed and applied to provide better medical diagnosis and treatment solutions.

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Conflicts of Interest: The authors declare no conflict of interest.

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