Emerging methods in therapeutics using multifunctional nanoparticles

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Abstract

Clinical translation of nanoparticle-based drug delivery systems is hindered by an array of challenges including poor circulation time and limited targeting. Novel approaches including designing multifunctional particles, cell-mediated delivery systems, and fabrications of protein-based nanoparticles have gained attention to provide new perspectives to current drug delivery obstacles in the interdisciplinary field of nanomedicine. Collectively, these nanoparticle devices are currently being investigated for applications spanning from drug delivery and cancer therapy to medical imaging and immunotherapy. Here, we review the current state of the field, highlight opportunities, identify challenges, and present the future directions of the next generation of multifunctional nanoparticle drug delivery platforms.

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1 INTRODUCTION

Research aimed at the development of novel nanoparticle (NP) technologies and their prospective use in a variety of medical applications have grown exponentially in recent decades. While broadly defined as colloidal particles ranging in size from nanometers into the submicron range, the scope of their properties, modes of preparation, compositions, and architectures vary vastly. As a result, their potential impact in numerous biomedical applications including drug delivery, tissue engineering, and diagnostics has become increasingly evident. Despite a wide range of tangible efforts, few NPs have had meaningful clinical impact. In fact, during the 20 years following the FDA approval of Doxil in 1995 for the treatment of Kaposi's sarcoma, fewer than 50 nanomedicines have received FDA approval

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(Bobo, Robinson, Islam, Thurecht, & Corrie, 2016). Recently published reviews (Anselmo & Mitragotri, 2016; Ventola, 2017) highlight current clinical trials of NP formulations while describing challenges impacting their successful translation to the clinic.

NP-based drug delivery systems aim to provide several advantages over their free drug counterparts including: (a) protection of loaded cargo from degradation or deactivation, (b) potential controlled release mechanisms, and (c) altered pharmacokinetics and specific control of biodistribution (Cheng, Al, Hui, Muzykantov, & Tsourkas, 2012; Peer et al., 2007). Despite their great promise, NPs suffer from rapid clearance from circulation, inefficient delivery to target tissues, and limited ability to cross challenging biological barriers such as the blood–brain barrier (BBB) (Agrahari, Agrahari, & Mitra, 2017; Anselmo & Mitragotri, 2014a; Blanco, Shen, & Ferrari, 2015). Therefore, the development of alternative drug delivery designs has proven essential to address the abovementioned hurdles.

In response to the challenge to navigate, alter, or interact with complex biological, physiological, or pathological processes, NP designs and architectures have evolved, in an attempt to address these challenges. However, while one class of particle or material may address a single barrier, it is unlikely to address them all. For example, in the case of drug delivery for cancer therapy, the bulk and surface properties best suited for this multi-step process—including systemic transport, tumor localization, cellular uptake, and effective drug release—are conflicting (Zou, Wang, & Feng, 2015). Approaches to address this conundrum include the development of multifunctional particles, cell mediated transport mechanisms, and the use of biologically derived materials. Here, we discuss recent advances in the development of such particles, their potential applications with a particular focus on drug delivery and persisting challenges in the field of multifunctional drug delivery carriers.

2 MULTIFUNCTIONAL NPs

Multifunctional particles can be defined as any particle system with two or more engineered properties. Here, we focus on two distinct types of multifunctional particles—(a) those with surface anisotropy and (b) those with bulk anisotropy. In the case of surface anisotropic particles, the bulk composition is often uniform and controlled, postfabrication surface modifications are used to create nonuniform surface features that diverge from their bulk properties. Conversely, bulk anisotropic particles contain multiple, distinct volumes within a single particle, often comprised of different materials, and as a result have dissimilar bulk properties. Discussed here, a variety of fabrication methods within each class, have been developed.

2.1 Surface anisotropy

Isotropic particles synthesized through a variety of methods can have anisotropic surface properties that are controlled by postmodification techniques. This can be achieved through the utilization of masks or templates, to controllably restrict the regions of particles to be modified (Hong, Jiang, & Granick, 2006; Paunov & Cayre, 2004). Interfaces (liquid–liquid (Zhang, Jin, & Zhao, 2009), liquid–solid (Takahara et al., 2005), air–liquid (Petit, Manaud, Mingotaud, Ravaine, & Duguet, 2001), and air–solid (Jang, Choi, Heo, Lee, & Yang, 2008), where particles are either mechanically placed or spontaneously accumulate, act to mask a portion of each particle while surface modifications are performed within a single phase of a two-phase system (Figure 1a). In other instances, the close packing of particles during the process, as in glancing angle deposition, self-imposes restraints on the surface areas of particles available for modification due to shadowing effects caused by neighboring particles (Figure 1c) (Pawar & Kretzschmar, 2009; Zhao, Ye, Wang, & Lu, 2002). Here, it is through the control of the deposition angle that dictates the surface area and pattern achieved. Processes such as physical deposition including etching (Nikravan, Haddadi-Asl, & Salami-kalajahi, 2018), chemical vapor deposition (Liang, Jordahl, Ding, Deng, & Lahann, 2015; Shepard et al., 2015), or lithography (Yake, Snyder, & Velegol, 2007) are used to selectively modify the exposed surfaces producing "patchy" particles. Finally, there are instances where uniform modification of the surface is performed to impart dissimilar particle characteristics from the bulk material (Figure 1b). In all cases, the result is a particle with regions of their surface with varied chemical, electrical, or amphiphilic properties distinct from their bulk properties. Together, these varied properties can be used to selectively modify particle surfaces. Examples with biomedical relevance include the covalent attachment of ligands for systemic targeting (Yake, Zahr, Jerri, Pishko, & Velegol, 2007) or PEGylation to alter particle pharmacokinetics (Rahmani et al., 2015).

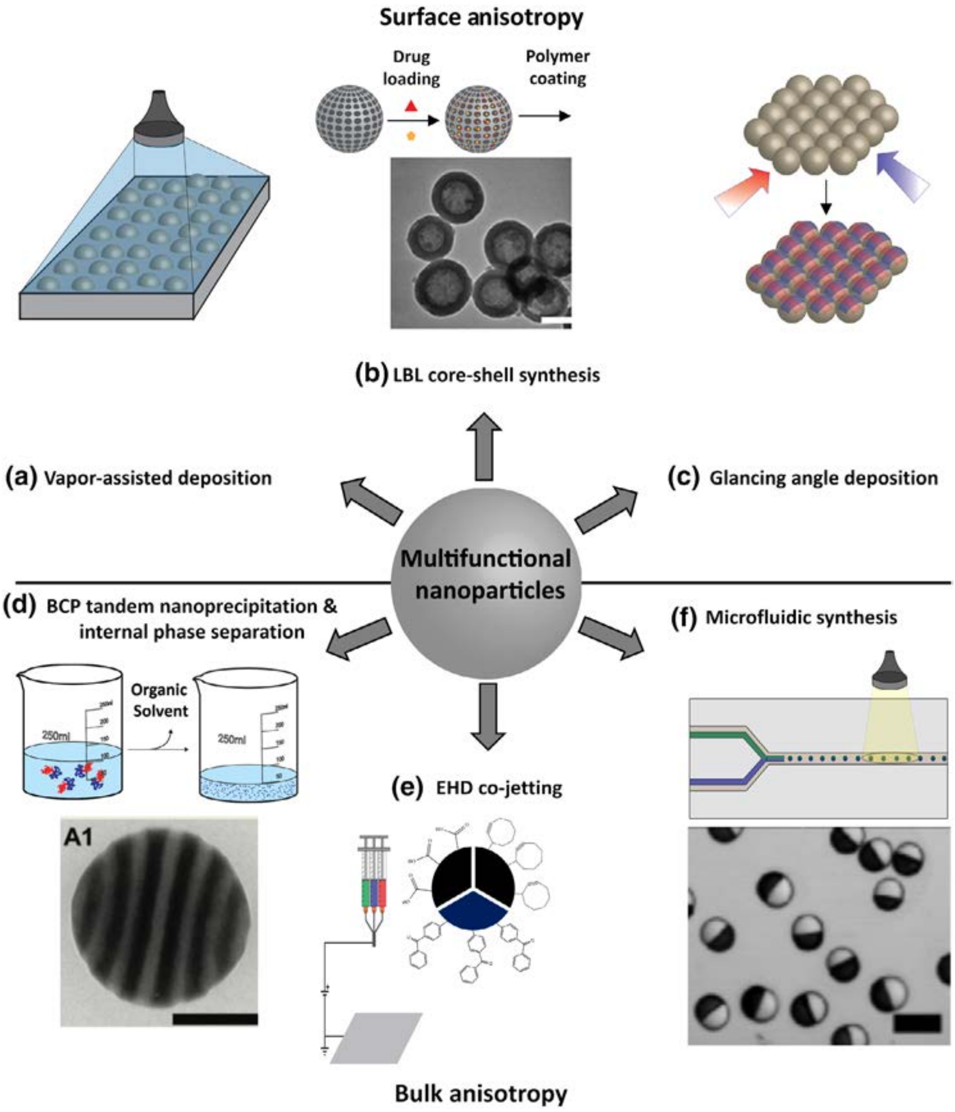

FIGURE 1 Techniques for the synthesis of multifunctional nanoparticles. (a) Vapor assisted deposition of macromolecules to select areas of nanoparticles through matrix assisted pulsed laser evaporation. Scale bar, 200 nm. Adapted with permission from Shepard, Christie, Sosa, Arnold, and Priestley (2015). (b) Layer by layer (LBL) fabrication of polymer coated, hollow silica nanoparticles for temporally controlled release of encapsulated drugs. Scale bar, 100 nm. Adapted with permission from Palanikumar et al. (2017). (c) Anisotropic, multifunctional patchy nanoparticles formed through the use of glancing angle deposition). Scale bar, 2 μm. Adapted with permission from Pawar and Kretzschmar (2009). (d) Tandem nanoprecipitation and internal phase separation employed to create surface reactive, patchy nanoparticles prepared through the use of block copolymers (BCPs) and tuning of preparation conditions. Scale bar, 100 nm. Adapted with permission from Varadharajan, Turgut, Lahann, Yabu, and Delaittre (2018). (e) Surface reactive, multicompartmental particles fabricated using electrohydrodynamic (EHD) cojetting through the spatially controlled addition of chemically orthogonal surface functional groups. Adapted with permission from Rahmani et al. (2014). (f) Continuous and high throughput synthesis of multicompartmental nanoparticles through the formation of compound droplets in flow and subsequent ultraviolet initiated crosslinking. Scale bar, 100 nm. Adapted with permission from Nie, Li, Seo, Xu, and Kumacheva (2006)

One of the more popular approaches for biological applications is the attachment of biomolecules to inorganic particles such as gold, or mesoporous silica that would otherwise have no targeting properties and lack biocompatibility. López et al. make use of a wax-in-water Pickering emulsion—a solid–liquid interfacial template—to create asymmetrically decorated mesoporous silica particles (Lopez et al., 2017). Effective delivery to cancer cells is achieved through targeting of cell membrane folate receptors before binding to mitochondria upon cellular uptake, finally delivering the encapsulated drug topotecan. By selectively controlling ligand placement, specific ligand density in each region is optimally maximized. Here, it is with a proper selection of ligands that aims to specifically interact with the cells of interest, namely folic acid, that facilitates increased tumor targeting and subsequent mitochondria binding while releasing the encapsulated payload.

Alternatively, surface modifications can increase cellular interactions and uptake through a more general approach of controlling surface chemistry and thereby affecting surface charge density. Recently, the controlled modification of cationic dendrimers with polyethylene glycol (PEG) has been used for targeting cartilage cells to treat osteoarthritis (Geiger, Wang, Padera, Grodzinsky, & Hammond, 2018). Geiger et al. make use of the highly controllable size and reactive surface amine groups to optimize surface charge, maximizing cellular uptake while minimizing toxicity, through the subsequent attachment of PEG. After attachment of the targeting ligand insulin-like growth factor 1 (IGF-1), these particles show increased specific uptake in cartilage cells, minimal toxicity, and significantly reduced disease symptoms.

2.2 | Bulk anisotropy

In contrast to the postmodification routes that are used to create multifunctional NPs with surface anisotropy, one or more bulk materials with distinct properties are used to create compositionally anisotropic particles. For example, the synthesis of complex NPs through controlled self-assembly processes can be achieved using block copolymers and variations in the solvent mixture (Figure 1d) (Shimoda et al., 2012; Varadharajan et al., 2018). On a larger scale, the selective surface functionalization of smaller building block particles can result in the formation of more complex supracolloidal assemblies (Castro, Constantin, Davidson, & Abécassis, 2016; Song et al., 2017). The production of liposomes (Morton et al., 2014; Reddy et al., 2016) or disk shaped particles (Guo et al., 2018; Kuai et al., 2017) made of amphiphilic molecules such as lipids can be formed through similar self-assembly processes. In contrast, flow processes, including microfluidics (Figure 1f) (Nie et al., 2006; Yuet, Hwang, Haghogooie, & Doyle, 2010) and electrohydrodynamic (EHD) cojetting (Figure 1e) (Cao, Wang, Wang, & Lou, 2014; Hwang et al., 2010; Roh, Martin, & Lahann, 2005; Wang, Zhang, & Wang, 2013), utilize the controlled flow of polymer solutions in specific orientations to one another in combination with a method of solidifying the individual particles to form stable colloids. Depending on the orientation of the individual flows, the distinct regions within the resulting particles can be radially anisotropic as is the case with core-shell particles (Cha et al., 2014; Duarte, Ünal, Mano, Reis, & Jensen, 2014), or adjacent to one another giving rise to a Janus structure (Nie et al., 2006; Roh, Martin, & Lahann, 2006). In the latter case, the bulk anisotropy directly translates to a surface anisotropy, which in some cases can be further modified for application specific properties. Alternatively, by taking a layer-by-layer approach, NPs can be assembled in a stepwise fashion, again resulting in layered particles with homogeneous surfaces (Deng et al., 2013; Liu et al., 2018; Nahire et al., 2013; Palanikumar et al., 2017). In each of the aforementioned processes, particles with chemically distinct regions and properties can be synthesized. Upcoming examples highlight how research groups have leveraged bulk anisotropic particles to create multifunctional particles with potential medical applications.

NP research has long been applied for the delivery of therapeutics and biomedical imaging. Particle-based imaging can be achieved via the addition of fluorescent dye molecules, nuclear imaging agents for positron emission tomography–computed tomography (PET/CT), single photon emission computed tomography (SPECT) (Devaraj, Keliher, Thurber, & Nahrendorf, 2009), and magnetic resonance imaging (MRI) (Liu et al., 2011; Reynolds et al., 2000), or encapsulated inorganic NPs for surface-enhanced Raman spectroscopy (SERS) (Strozyk et al., 2017) imaging. The use of multi-compartment particles may allow for these to be incorporated into a single particle system and coupled with controlled release of drugs. Combining the delivery and release of therapeutics while also providing a method of monitoring biodistribution and intracellular fate, termed theranostics, can prove to be a valuable tool within the clinic. Misra et al. demonstrated the ability to create biphasic NPs comprising of a poly(lactic-co-glycolic acid) (PLGA) compartment loaded with an imaging agent alongside a second pH-sensing, small interfering RNA (siRNA)-loaded compartment (Misra, Bhaskar, Clay, & Lahann, 2012). The synthesized particles demonstrated not only the ability to serve the dual function of particle tracking and therapeutic release but also made use of significant swelling of a single hemisphere to facilitate endosomal escape.

The synthesis of NPs with bulk anisotropy lends itself to the development of multifunctional particles with a unique control over their interactions with other particles or biological systems. For example, the use of dissimilar pH responsive polymers to form distinct regions within a single NP can be used to individually load and tune the release of encapsulated cargo (Hwang & Lahann, 2012; Rahmani et al., 2016). Gröschel et al. made use of block copolymers to create patchy particles capable of guided self-assembly to form supracollodial hierarchical assemblies (Gröschel et al., 2013). In contrast, Varadharajan et al., also working with block copolymers, recently employed tandem nanoprecipitation and internal phase separation techniques to produce NPs with complex structural and chemical anisotropy (Varadharajan et al., 2018). The resulting particles and their bulk morphology was dependent on solution parameters including polymer concentration and solvent ratios. Variations in these conditions resulted in biphasic anisotropic particles with onion-like, dotted, or lamellar patterns where each surface region could then potentially be selectively modified. Such patterned particles could have future biomedical applications including biosensors through coenzyme immobilization.

Creating bulk anisotropic Janus particles allows for selective and controlled modifications to be performed on the surface. Rahmani et al. demonstrated this through the synthesis and subsequent surface modification of tri-compartmental particles (Rahmani et al., 2014). Here, a similar PLGA base was used in combination with dopants of functional polylactic acid (PLA) polymers. It was shown that by incorporating small amounts of a functional polymer within the bulk of an otherwise isotropic particle system, controlled surface functionalization through orthogonal click chemistry reactions could be used to selectively decorate the particle surface. This approach allows for the covalent attachment of specific targeting or stealth moieties with control over density, placement, and relative orientation of individual ligands relative to one another. Furthermore, the adaptability of the process suggests that the number of compartments and attached ligands is limited only by the number of orthogonal chemistries that can be performed on the resulting particle.

While the highlighted methods of multifunctional NPs aim toward overcoming biological barriers in the field of drug delivery, a great deal of progress remains to be made. Of particular importance is the ability to translate optimal cell penetrating and drug delivery achieved within in vitro systems to clinical relevance. The most daunting of challenges involves maintaining favorable particle attributes for cellular uptake while minimizing in vivo clearance from circulation to maximize targeting capabilities. For years, the gold standard of surface modification, PEGylation, promised to be a means to add a stealth-like quality to nano-sized colloids in the bloodstream. However, even to date, the fractions of injected particles remaining in circulation over extended periods of time, while improved, remain disappointing using this method (Rahmani et al., 2015). More concerning is the recent observation of circulating antibodies against PEG as an innate immune response (Garay, El-Gewely, Armstrong, Garratty, & Richette, 2012; Zhang, Sun, Liu, & Jiang, 2016). Together, these results motivate current research to identify alternative means to extend particle circulation, reduce their rapid clearance, increase local targeting, and effectively penetrate biological barriers such as BBB.

3 CELL-MEDIATED DELIVERY OF NPs

Circulatory cells, as the body's own delivery vehicles, possess inherent abilities specifically long circulation times, natural tissue targeting, and the ability to cross impermeable barriers. These significant properties make them great candidates to address some challenges concerning NP drug delivery systems (Anselmo & Mitragotri, 2014a; Ayer & Klok, 2017). One such delivery systems, termed "cellular hitchhiking" is an enhancement of the traditional ones, wherein targeted delivery via body's natural vehicle, that is, circulatory cells and optimal release of the cargo from engineered NPs are realized in one delivery platform.

Cellular hitchhiking has been performed using a variety of cell types (see Table 1). In this review we focus on red blood cells (RBCs), leukocytes, and stem cells, all of which have been exploited for the cell-mediated transport of NPs. We furthermore elaborate on various strategies that have been used to incorporate NPs into or conjugate them onto the surface of these circulatory cells.

3.1 Red blood cells

Constituting >99% of total blood cells, RBCs are long-circulating cells with a lifespan of 100–120 days in humans and natural carriers of many substances, especially oxygen, in the blood stream (Muzykantov, 2010). The innate properties of RBCs, such as a long circulation time, reversible deformation, and ability to squeeze through capillaries smaller than their diameter (Anselmo & Mitragotri, 2014a; Tomaiuolo, 2014) make them suitable candidates as platforms for drug delivery systems (Muzykantov, 2010; Pang et al., 2017).

In general, there exists two main methods to obtain RBC-mediated NP drug delivery systems: (a) to internally load the NPs into RBCs, or (b) to attach them onto the surface of the cells. Wu et al. fabricated RBC-based micromotors, wherein iron oxide NPs were encapsulated into the RBCs and the motors were powered and activated by ultrasound and an applied magnetic field, respectively (Wu et al., 2014). Encapsulation of cargoes into RBCs using hypotonic dilution methods requires the formation of transient pores in the RBC membrane for diffusion of NPs into cells (Wu et al., 2014)

TABLE 1 Advantages, limitations, and examples of in vivo applications of cellular hitchhiking formulations

Hitchhiked cell type	Cell type advantages	Cell type limitations	Particle cargo	Benefits of cellular hitchhiking	References
Red blood cells	Abundant Long circulation time Easy isolation	Limited tissue targeting	200 nm spheres and rod shape polystyrene particles	Increased lung targeting	Anselmo et al., 2015; Ding, O'Banion, Welfare, and Lawrence, 2018
Macrophages/ monocytes	Ability to phagocytose nanoparticles Cross biological barriers Naturally migrate to sites of inflammation Reach hypoxic areas of tumors	Low drug loading efficiency Endosomal degradation of phagocytosed cargo	Self assembled polyethyleneimine poly(ethylene glycol) catalase	Enhanced delivery of catalase to PD affected brain regions (crossing blood brain barrier)	Anselmo and Mitragotri, 2014a; Batrakova et al., 2007
T cells	Ability to target specific cells Dual carrier and therapy capability	Difficult harvesting and handling Short in vivo lifespan	300 nm multilamellar lipid nanoparticles loaded with IL 15 and IL 21	Enhanced tumor elimination in established B16 melanomas	Singh and Mitragotri, 2019; Stephan, Moon, Um, Bersthteyn, and Irvine, 2010
Stem cells	Ability to internalize nanoparticles Tumoritropic migratory ability	Difficult isolation and expansion Reports of MSCs association with promoting primary and metastatic tumor growth	Poly(ethylene glycol) poly(diisopropyl amino) ethyl methacrylate nanoparticles loaded with docetaxel	Enhanced tumor delivery due to improved migration to hypoxic tumor cores in a triple negative breast cancer (TNBC) mouse model	Corsten and Shah, 2008; Mooney et al., 2014

making it more invasive in comparison to anchoring the cargoes on their surface (Villa, Cines, Siegel, & Muzykantov, 2017; Villa, Seghatchian, & Muzykantov, 2016). Surface loading can be achieved via nonspecific binding (electrostatic, van der Waals, hydrogen bonding, and hydrophobic forces) (Chambers & Mitragotri, 2007), or specific binding (ligand–receptor interactions or chemical conjugation) (Villa, Anselmo, Mitragotri, & Muzykantov, 2016; Villa, Seghatchian, & Muzykantov, 2016).

Adsorption of NPs onto RBCs surfaces has been explored as a means of avoiding rapid clearance by the reticuloendothelial system (RES) (Chambers & Mitragotri, 2004; Chambers & Mitragotri, 2007). As an example, RBC-hitchhiking of model polystyrene NPs led to a 100-fold increase of NPs in vivo circulation time (Chambers & Mitragotri, 2007). Because surface adsorbed NPs will eventually detach from carrier RBCs due to cell–cell interaction and shear forces, engineering the detachment of NPs and their transfer to microvasculature endothelium will enable targeted organ delivery using RBC hitchhiking (Anselmo et al., 2013; Anselmo, Kumar, et al., 2015; Brenner et al., 2018). In a recent study, NPs adsorbed onto RBCs were delivered to the first microcapillary bed that the RBC–NP conjugates encountered downstream to their injection site (Figure 2a). Selective placement of intravascular catheters upstream of specific organs delivered RBC-hitchhiked NPs to various target organs such as lung, kidney, and brain. RBC-liposome conjugates injected intravenously showed an increased brain delivery of 11.5% of the injected dose (Brenner et al., 2018) compared to transferrin-targeted NPs having 1% target rates, at best (Wiley, Webster, Gale, & Davis, 2013). It is important to optimize the loading ratio of NPs onto the RBCs in order to provide optimal delivery but not induce adverse effects on the carrier cells (Pan et al., 2018). To this end, Pan et al. designed high-throughput in vitro assays to characterize the sensitivity of hitchhiked RBCs to potential damage of adsorbed NPs (Pan et al., 2016).

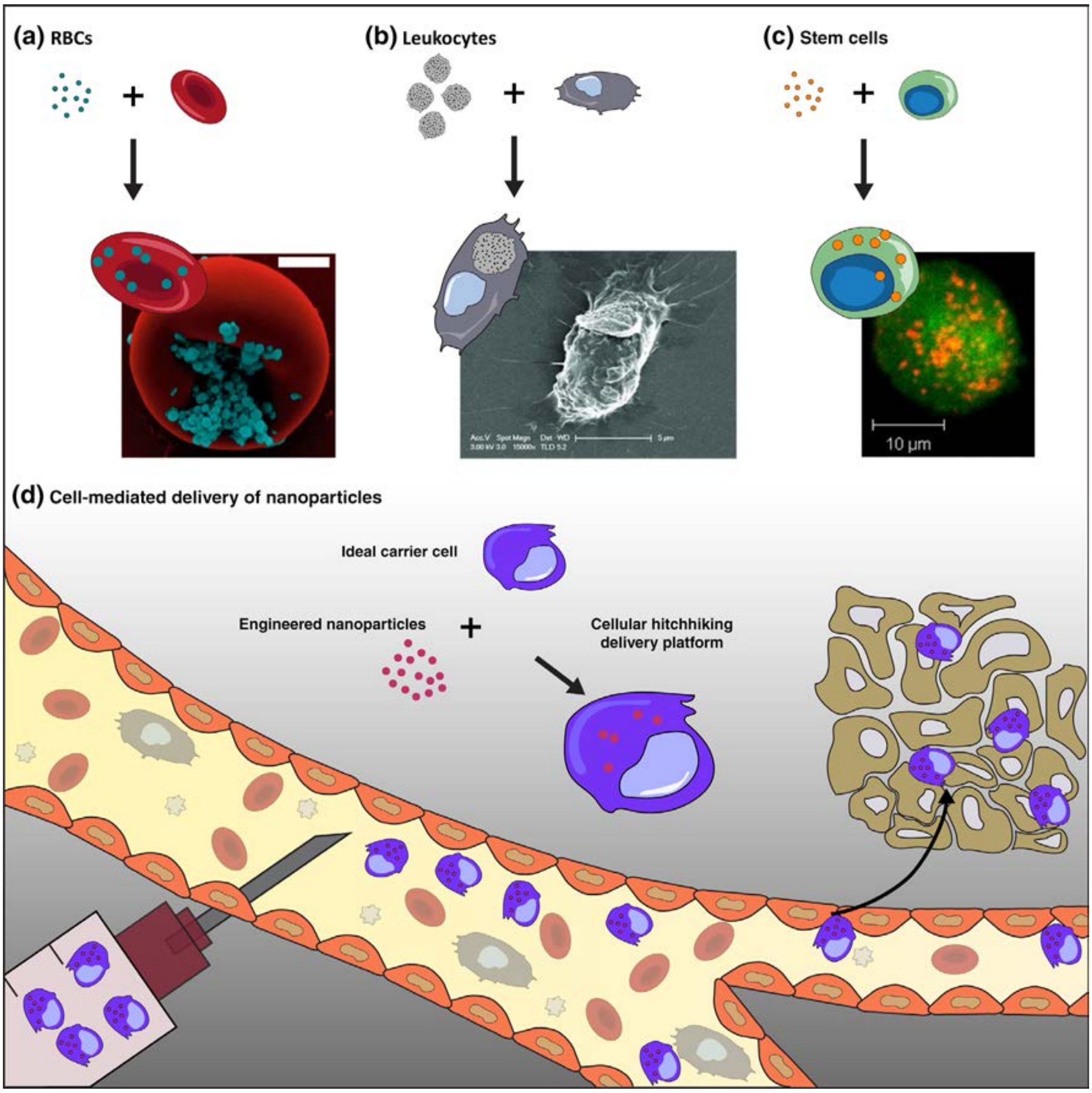

FIGURE 2 Different circulatory cells used in cellular hitchhiking formulations. (a) Scanning electron micrographs of nanogels adsorbed onto the surface of murine red blood cells (RBCs) in vitro. Scale bar 1 μm. Adapted with permission from Brenner et al. (2018). (b) Scanning electron micrographs of hyaluronic acid coated backpack attached to the surface of J774 mouse macrophages after 3 hr incubation in cell culture conditions. Scale bar 5 μm. Adapted with permission from Doshi et al. (2011). (c) Confocal image of fluorescently labeled nanoparticles conjugated to biotinylated neural stem cell stained with calcein AM. Scale bar 10 μm. Adapted with permission from Mooney, Weng, et al. (2014). (d) Schematic drawing of circulatory cell mediated targeting and delivery of nanoparticles

3.2 Leukocytes

Serving as major components of the adaptive and innate immune system, leukocytes are responsible for fighting inflammation, infection, and tumor growth (Grivennikov, Greten, & Karin, 2010; Sahdev, Ochyl, & Moon, 2014). Leukocytes inherently migrate to areas hard-to-reach by traditional NPs such as inflamed tissue (Nourshargh & Alon, 2014), migrate across endothelial barriers (Gordon & Taylor, 2005), and reach the hypoxic area of tumors (Lewis & Murdoch, 2005; Murdoch, Giannoudis, & Lewis, 2004), and thus are an attractive cell choice for hitchhiking (Pang et al., 2017).

Macrophages and monocytes as phagocytic cells can naturally internalize NPs and carry them to target sites that are otherwise largely inaccessible (Evans et al., 2018; Moore et al., 2017; Si, Shao, Shen, & Wang, 2016). For example, macrophages have been used for delivering various nanocarriers across BBB such as self-assembled polyethyleneimine-poly (ethylene glycol) catalase in a Parkinson's disease model (Batrakova et al., 2007), and gold-silica nanoshells for photothermal therapy for glioma in vitro (Amani, Makkouk, & Sun, 2011) and in vivo (Madsen et al., 2015). In another study, mouse peritoneal macrophages loaded with liposome-doxorubicin were delivered to tumors both in subcutaneous and metastasis xenograft tumor models (Choi et al., 2012). The macrophages were viable for up to 12 hr in spite of the time-dependent release of doxorubicin from the liposomes (Choi et al., 2012). After internalization, NPs are subject to endosomal degradation (Blanco et al., 2015) that can cause premature drug release, reducing the therapeutic effect (Batrakova et al., 2011; Blanco et al., 2015; Doshi et al., 2011) or affecting the migration of the carrier cells (Jiang, Yu, Yen, & Woo, 2015; Klyachko et al., 2017). To overcome these challenges, NPs that can be immobilized on cell surfaces while avoiding phagocytosis were proposed (Figure 2b) (Doshi et al., 2011; Swiston et al., 2008; Swiston, Gilbert, Irvine, Cohen, & Rubner, 2010). Klyachko et al. showed that "backpacks" loaded with a potent antioxidant, catalase, were attached to the surface of macrophages and transmigrated across inflamed BBB in a mouse model of LPS-induced encephalitis (Klyachko et al., 2017). Alongside macrophages, a typical feature of monocytes as circulatory cells to migrate toward inflammation sites along a chemoattractant gradient (Deshmane, Kremlev, Amini, & Sawaya, 2009) made them suitable to carry particles to inflamed tissues. Anselmo et al. took advantage of IgG-Fc receptor interactions to attach cellular "backpacks" on the surface of monocytes while avoiding phagocytosis due to the polymeric backpacks size, disc like shape, and flexibility. Cellular functions such as transmigration through endothelium, or differentiation into macrophages were unimpaired after attachment of the backpacks onto monocytes. Monocyte-hitchhiked backpacks showed a ninefold higher accumulation in the inflamed skin compared to noncell attached backpacks and a twofold higher targeting of inflamed lungs than to normal lungs (Anselmo et al., 2015).

T cells as key components of adaptive immune system are capable of sensing danger signals from invading pathogens and cancer. Upon antigen presentation, tumor specific T cells become activated to eliminate tumor cells (Restifo, Dudley, & Rosenberg, 2012). In the context of adoptive T cell-based strategies, utilizing patient's natural T cells or engineered T cells with chimeric antigen receptors to mediate tumor cell eradication has suggested promising new directions (Restifo et al., 2012). However, one of the major barriers for cell-based therapies is loss of transplanted cell viability and function. Showing the enhancement of cell therapy outcome, Stephan et al. reported the immobilization of adjuvant drug-loaded NPs to the surface of therapeutic cells via maleimide–thiol conjugation to provide sustained pseudoautocrine stimulation of the transferred cells in vivo (Stephan et al., 2010). Sustained release of the interleukins (IL-15 and IL-21) from the conjugated NPs mediated robust T-cell proliferation in vivo and resulted in enhanced eradication of established B16 melanomas (Stephan et al., 2010). Because of tissue-homing ability of T lymphocytes, they were selected as carriers for lipid nanocapsules loaded with potent topoisomerase I poison SN-38 (Huang et al., 2015). The nanocapsules were covalently attached to the surface of polyclonal T lymphocytes to deliver the drug into lymphoma tumors. This approach reduced tumor growth significantly and increased survival compared to free SN-38 and SN-38 loaded lipid nanocapsules alone (Huang et al., 2015). Cellular hitchhiking can be taken one step further if the cells play a dual role of transport and therapy. An interesting proof-of-concept study was reported by Wayteck et al. which cytotoxic T cells were selected due to both their tumoritropic migratory properties and innate tumor cell killing ability to carry siRNA loaded liposomes (Wayteck et al., 2016).

3.3 Stem cells

Stem cell therapy often considered to be vital for tissue engineering and regenerative medicine (Fliervoet & Mastrobattista, 2016). A more recent development is the use of specific stem cell lineages such as mesenchymal stem cells (MSCs) and neural stem cells (NSCs) for drug delivery applications (Tan, Wu, Zhang, & Zhang, 2015). Their tumoritropic migratory nature make them desirable for targeted delivery of therapeutics (Corsten & Shah, 2008; Mimeault, Hauke, & Batra, 2007) and multimodality imaging agents (Huang et al., 2013) in cancer therapy.

For designing stem cell-mediated delivery platforms, NPs can be loaded onto the cells surface as a first approach. For example, doxorubicin-loaded nanorattles were surface decorated with anti-CD73 or anti-CD90 to anchor to MSCs through antibody–antigen interaction (Li et al., 2011). These conjugates were able to migrate toward the glioma xenograft and resulted in enhanced tumor cell apoptosis compared to the free drug and the drug-loaded silica nanorattles (Li et al., 2011). In another example, NSCs were chosen as carries due to their ability to overcome high interstitial pressures and penetrate to hypoxic tumor regions. The NSCs mediated the transport of docetaxel-loaded NPs to enhance the efficiency of intratumorally administered NPs. A pH sensitive bond was used to conjugate NPs to the surface of NSCs via biotin-streptavidin interaction (Figure 2c). This hybrid cell–NP system showed enhanced distribution and retention of the NPs in a tripe negative breast cancer mouse model (Mooney, Weng, et al., 2014).

In a second approach to formulate stem-cell based delivery platforms, NPs can be encapsulated into the stem cells. Polylactic acid NPs and lipid nanocapsules were internalized by marrow-isolated adult multilineage inducible (MIAMI) cells, a subpopulation of MSCs as potential NP carriers in brain tumor therapy. It was shown that after their direct tumoral injection, loaded MIAMI cells migrated and distributed around the tumor mass (Roger et al., 2010). In another example, gold nanorods (AuNRs) taken up by NSCs demonstrated potential in improvement of photothermal therapy efficiency (Mooney, Weng et al., 2014). When the AuNRs were transported by NSCs after intratumoral injection, broader and more homogenous distribution of AuNR within the tumor was observed compared to free AuNRs. Improved in vivo delivery of AuNRs mediated by NSCs resulted in reduced tumor recurrence rates after NIR exposure (Mooney, Roma, et al., 2014).

When designing efficient cell-mediated NP delivery systems for targeting specific organs, a rational selection of appropriate cells is as important as precisely tuning the properties of NPs. Nonetheless, challenges to this approach include sufficient drug loading capacity, premature drug release, triggered controlled release, preservation of the drug cargo from intracellular degradation, and protection of cell carriers from drug cytotoxic effects. Moreover, cost barriers and sufficient harvesting, or expanding of cells without contamination for reinjection into the body, and efficient migration of cell carriers to the target site are other important concerns. Although there are challenges that need to be addressed, cell-mediated delivery platforms offer promising opportunities in improving diagnosis and therapeutics for various chronic diseases such as cancer. Developing smart biomaterials, engineering particle design parameters, and utilizing deliberate methods to conjugate NPs to suitable cells can address some of the above-mentioned challenges.

4 PROTEIN NPS

A recent development in the field of NP-based drug delivery replaces synthetic polymers with proteins as the primary building blocks of NPs. As a material, proteins show great promise due to their variety, function, design flexibility through genetic engineering, improved biocompatibility and potentially lower immunogenicity. Three main techniques to develop protein NPs (PNPs) will be explored in this review: NP albumin bound (*nab*) technology, self-assembly, and coacervation.

4.1 Nab technology

Nab technology is one of the oldest and most developed methods for making PNPs. Developed by Abraxis Bioscience (now a part of Celgene) to create a way of delivering paclitaxel, *nab*-technology forces highly hydrophobic drugs into the internal hydrophobic pockets of human serum albumin (HSA) using a high-pressure manufacturing process (Figure 3a). Paclitaxel is normally administered using harsh organic solvents (Singla, Garg, & Aggarwal, 2002). By packaging the drug in albumin, a common protein in human blood that is not only water soluble but also has a naturally long circulation time, the drug can be delivered with reduced side effects (Desai, 2007). The first FDA approved *nab* product was Abraxane, which has been approved for use as a first line therapy for nonsmall cell lung cancer, metastatic adenocarcinoma, and as treatment for metastatic breast cancer. Additionally, Abraxane is in Japanese clinical trials by Celgene for use in metastatic pancreatic cancer and gastric metastatic cancer.

In addition to the success of Abraxane, multiple other *nab* technologies are under investigation at both the industrial and academic level. ABI-008 through ABI-011 are a family of *nab* based drugs that are undergoing clinical trials. For example, AB-009 (*nab*-rapamycin/sirolimus, brand name Tarzifix) is under investigation by Aadi Bioscience (licensed from Celgene) in a variety of Phase 1 and 2 trials ranging from metastatic colorectal cancer to pulmonary arterial hypertension (see Table 2). In addition to multiple clinical trials, next generation *nab* technologies are actively being investigated. For example, actively targeted variants of *nab* particles have been made. Thao et al. developed *nab* particles made out of lactosylated albumin loaded with a mixture of paclitaxel and doxorubicin. The particles were designed to take advantage of the high affinity of lactose to asialoglycoprotein receptors, which are overexpressed in hepatocellular carcinomas. The particles were shown through in vitro and in vivo experiments to have increased accumulation in liver versus control (Pac/dox loaded into naïve albumin *nab* particles) (Thao et al., 2017). In addition to applying targeting moieties to *nab* particles, there has been work done on the use of adjuvants in potential therapies. An interesting case was the work performed by Kinoshita et al., where Abraxane was delivered with a S-nitrosated HSA dimers (Kinoshita et al., 2017). By modifying the HSA dimer through a disulfide bond on the free cysteine on albumin, they were able to release nitrous oxide (NO) as a vasodialator. This effect increased the delivery and efficacy of

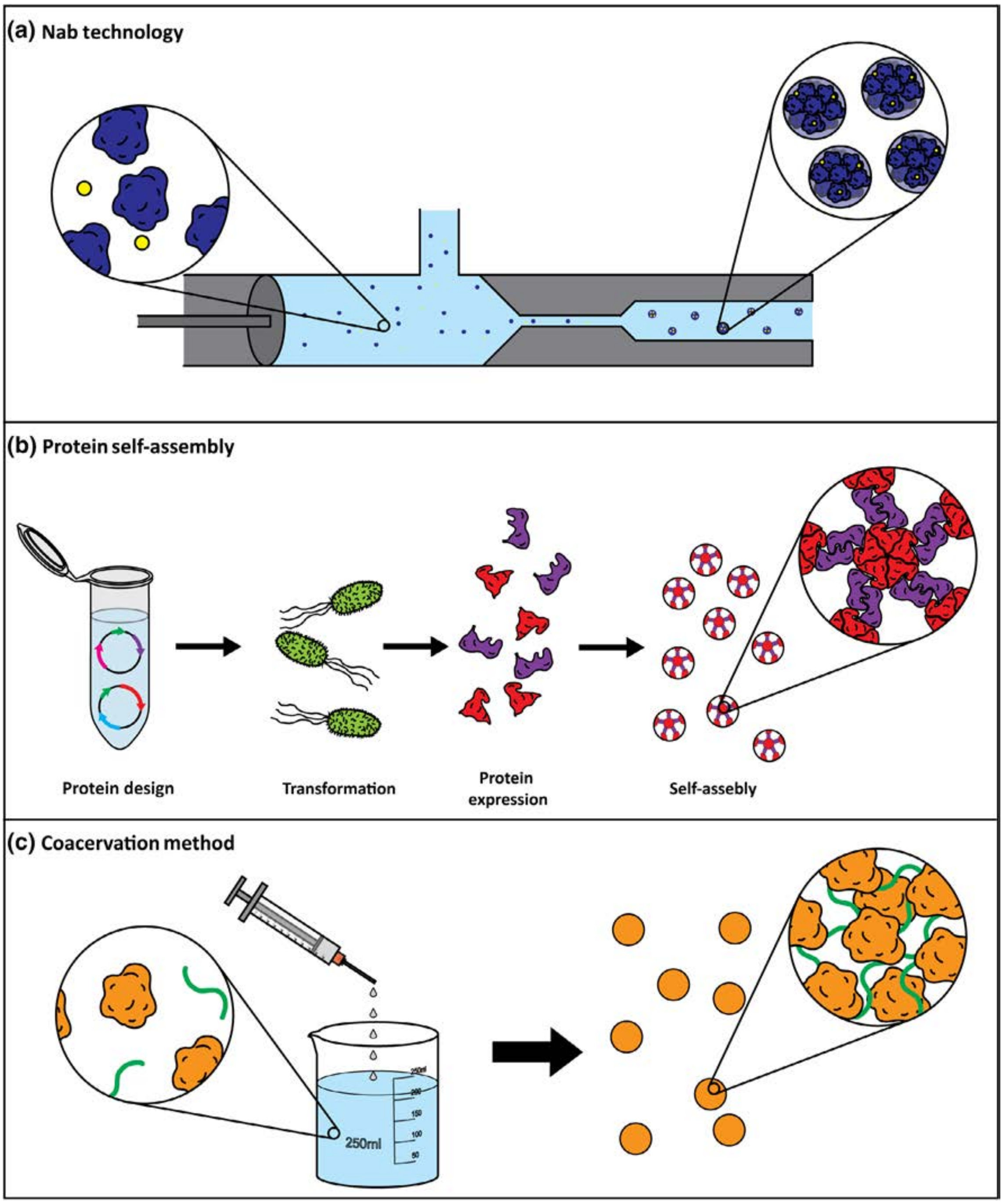

FIGURE 3 Protein Nanoparticles hold great promise in medicine due to their variety and inherent functionalities. Three main methods exist to synthesize these particles. (a) *Nab* technology works by using a sheer mediated process to force hydrophobic drugs within proteins and subsequently cause the proteins to aggregate into nanoscale particles. (b) Self assembly techniques use the expression of specially designed proteins by microorganisms that subsequently self assemble into structures that can be used for broad variety of therapeutic applications. (c) Coacervation functions by the addition of an organic solvent or reagent to a protein solution, which causes the formation of particles that are subsequently crosslinked using bifunctional crosslinkers

Abraxane in colon cancer and melanoma murine models, and reduced metastases. Creative combinations of therapies such as this show potential translatability in that they follow the pharmaceutical industry model of expanding the potential of a therapy through combination studies.

While showing great potential, *nab* technologies have potential downsides. Early work has shown that Abraxane is associated with more rapid plasma clearance compared to the traditional liposomal formulation of paclitaxel (Taxol)

(Sparreboom et al., 2005). Abraxane NPs are stable in ex vivo saline solutions, but the particles quickly break down into albumin–paclitaxel complexes following administration (Desai, 2007). This poor colloidal stability has been suggested as the reason behind the rapid clearance of the NPs (Ruttala & Ko, 2015a). Work has been done to improve the colloidal stability of *nab* particles (Ruttala et al., 2017; Ruttala & Ko, 2015a), but these works have used albumin bound paclitaxel particles made through coacervation techniques, not high-pressure homogenization as Abraxane and other *nab* particles are. Further studies are thus needed to substantiate claims of improved stability. This problem of using coacervation synthesized particles as a stand-in for Abraxane has also been seen in other studies with potentially impactful advances (Ruttala & Ko, 2015b; Tian et al., 2017). In addition to poor clearance profiles, *nab* technology has the inherent downside of harsh synthetic conditions (Desai, 2014). This potentially limits the use of *nab* technology to deliver active proteins, such as enzymes, in ways that other synthetic routes that are able to (Estrada, Chu, & Champion, 2014). Excellent reviews of *nab* technologies have been written by Hawkins et al. and Tan et al., among others, which we recommend for further reading (Hawkins, Soon-Shiong, & Desai, 2008; Tan & Ho, 2018). *Nab* technology has shown the clinical potential of proteins as nanocarriers in medicine.

4.2 Self-assembled protein NPs

Self-assembled NPs are nanoscale structures made of protein complexes that can self-assemble to form PNPs (Figure 3b). These structures are designed by creating recombinant proteins that contain oligomerization domains that create structure, and then a variety of other domains that can result in specific activity (Yeates, Liu, & Laniado, 2016). The synthetic methods and design strategies for nanoscale protein structures have been excellently summarized in recent reviews (Arai, 2018; Yeates et al., 2016).

An interesting application of self-assembled PNPs in the medical space is the use of caged protein NPs (Molino & Wang, 2014). These particles are made up of protein units that self-assemble under specific conditions into hollow cage-like structures. Inside these structures it is then possible to load a variety of therapeutic molecules such as enzymes (Kaczmarczyk, Sitaraman, Young, Hughes, & Chatterjee, 2011) and small molecules (Zhen et al., 2013). In a recent study, Kawakami et al. designed a 60-mer protein cage with a defined structure. Notably, they were able to design the particle so that specific residues faced either the exterior or interior of the cage, and subsequently were able to covalently modify these particles (Kawakami et al., 2018). These covalent modifications were done using disulfide bonds, and thus this system could be designed to carry a drug in the inside of the cage, and then be released in a reducing environment.

These self-assembled NP technologies are elegant, sophisticated, and complicated, but these very characteristics call to question their potential for translation into the clinic in the near future. Most of the proteins used in these NPs are not only novel recombinant proteins, but are also expressed in nonmammalian organisms such as *Escherichia coli* (Kawakami et al., 2018). Expression in nonhuman organisms of recombinant proteins presents many regulatory problems and costs, as has been shown though the past 30 years with the rise of recombinant antibody and antibody fragment (Fab) technology (Ecker, Jones, & Levine, 2015). Yet, with careful development, the rise of the multi-billion dollar biological therapeutics field shows the potential for progressively more sophisticated therapies to enter the market.

4.3 Coacervation-synthesized protein NPs

During coacervation, a "coacervation agent," usually an organic solvent such as acetone or ethanol, is added to a concentrated aqueous solution of a protein of interest. The coacervating agent dehydrates the proteins and causes the precipitation of NPs from the solution. The particles can then be crosslinked, rendering them water insoluble (Figure 3c). By controlling a variety of conditions, including the protein type, the rate of addition of the coacervating agent, the temperature of the procedure, the salt content of the solution, and the crosslinking agent and time, the resulting NP size, mechanical properties, and functionalities can be tailored to fit the needs of the application (Von Storp, Engel, Boeker, Ploeger, & Langer, 2012; Weber, Coester, Kreuter, & Langer, 2000). In addition, the process is highly reproducible, and the particles can be surface functionalized and loaded with a variety of therapeutics (Dreis et al., 2007; Herrera Estrada & Champion, 2015; Langer et al., 2008).

Initial work using coacervation focused on albumin proteins, but the field is now expanding to a variety of different proteins and applications. A wide variety of different proteins have been formulated into NPs, as detailed in a recent

TABLE 2 Past, current, or planned clinical trials of Abraxane-like *Nab* nanoparticles

Drug code	Active ingredient	Mechanism of action	Clinical trial phase	Trial description and indication	Trial outcome	Trial years	Sponsor(s)	Clinical trial number
ABI-008	Docetaxel	Chemotherapeutic, a semi-synthetic taxane analogue of paclitaxel	1/2	Single agent therapy of *nab*-docetaxel for hormone refractory prostate cancer	Completed	2007–2011	Celgene Corporation	NCT00477529
			1/2	Single agent therapy of *nab*-docetaxel for metastatic breast cancer	Terminated, reason not given	2007–2008	Celgene Corporation	NCT00531271
ABI-009	Rapamycin (aka Sirolimus)	mTOR inhibitor, FDA approved as small molecule drug for the prevention of organ transplant rejection and the treatment of lymphangioleiomyomatosis	1	Single agent therapy of *nab*-rapamycin for advanced nonhematologic malignancies	Completed	2007–2011	Celgene Corporation	NCT00635284
			1/2	Single agent therapy of *nab*-rapamycin for nonmuscle invasive bladder cancer	Ongoing	2014–2020 (planned)	Aadi, LLC and National Cancer Institute	NCT02009332
			2	Single agent therapy of *nab*-rapamycin for advanced perivascular epithelioid cell tumors (PEComa), malignancy with relevant genetic mutations or mTOR pathway activation	Ongoing	2015–2020 (planned)	Aadi, LLC	NCT02494570, NCT03817515
			1	Single agent therapy of *nab*-rapamycin for severe pulmonary arterial hypertension	Ongoing	2017–2020 (planned)	Aadi, LLC	NCT02587325
			1	Single agent therapy of *nab*-rapamycin for advanced cancer with mTOR mutations	Ongoing	2016–2019 (planned)	Mayo Clinic and National Cancer Institute	NCT02646319
			1	Combination therapy of *nab*-rapamycin, temozolomide, and irinotecan hydrochloride for pediatric patients with recurrent or refractory solid tumors	Ongoing	2017–2020 (planned)	Children's Oncology Group and National Cancer Institute	NCT02975882
			1/2	Combination therapy of Nivolumab and *Nab*-rapamycin for advanced sarcoma	Ongoing	2017–2020 (planned)	Sarcoma Oncology Research Center, LLC and Aadi, LLC	NCT03190174
			1/2	Combination therapy of *Nab*-rapamycin, FOLFOX and bevacizumab as first-line therapy for advanced or metastatic colorectal cancer	Ongoing	2018–2021 (planned)	Aadi, LLC	NCT03439462
			2	Single agent therapy of *nab*-rapamycin for recurrent high grade glioma and newly diagnosed glioblastoma	Ongoing	2018–2021 (planned)	Aadi, LLC	NCT03463265
			1	Single agent therapy of *nab*-rapamycin for surgically refractory epilepsy	Ongoing	2018–2019 (planned)	Seattle Children's Hospital and Aadi, LLC	NCT03646240
			1	Combination therapy of *Nab*-rapamycin, pomalidomide and dexamethasone for relapsed and refractory multiple myeloma	Planned	2018–2024 (planned)	Massachusetts General Hospital and Aadi, LLC	NCT03657420
			1/2	Combination therapy of *nab*-rapamycin and pazopanib hydrochloride for nonadipocytic soft tissue sarcomas	Ongoing	2019–2021 (planned)	University of Washington	NCT03660930

(Continues)

TABLE 2 (Continued)

Drug code	Active ingredient	Mechanism of action	Clinical trial phase	Trial description and indication	Trial outcome	Trial years	Sponsor(s)	Clinical trial number
			2	Single agent therapy of *nab*-rapamycin for metastatic, Unresectable, low or intermediate grade neuroendocrine tumors of the lung or Gastroenteropancreatic system	Ongoing	2018–2020 (planned)	Aadi, LLC and Ochsner Health System	NCT03670030
			2	Single agent therapy of *nab*-rapamycin for genetically-confirmed Leigh or Leigh-like syndrome	Planned	2019–2023 (planned)	Aadi, LLC	NCT03747328
ABI-010	Tanespimycin	Aka 17AAG a Hsp90 inhibitor analogue of geldanamycin currently under investigation for various indications	1	Combination therapy *nab*-paclitaxel and *nab*-17AAG for advanced nonhematologic malignancies	Withdrawn before enrolling its first participant	2012–2014	Celgene	NCT00820768
ABI-011	IDN 5404	Thiocolchicine analogue. A dimer shown to have vascular disrupting activity and to be a topoisomerase I inhibitor	1	Single agent therapy of *nab*-IDN 5404 for Advanced Solid Tumors or Lymphomas	Terminated, reason not given	2011–2014	Celgene Corporation	NCT01163071
			1	Single agent therapy of *nab*-IDN 5404 for advanced solid tumors or lymphomas	Ongoing	2017–2019 (planned)	NantBioScience, Inc.	NCT02582827

review (Fathi, Donsi, & McClements, 2018). These proteins have been used in applications such as the packaging of small molecules and micro-nutrients for the food industry. Guo et al. produced whey protein NPs loaded with zinc and showed that particle size could be controlled by modulating the amount of zinc added and the synthesis conditions. Additionally, they showed through in vitro experiments that site specific delivery of zinc was possible by pH dependent release of the micronutrient from the PNPs to its nutritionally relevant site, the intestinal track, as opposed to the stomach (Shao, Shen, & Guo, 2018).

Through coacervation techniques, PNPs have been prepared from a wide variety of polypeptides and proteins for therapeutic purposes (Chang, Stadmiller, Staskevicius, & Champion, 2017; Estrada et al., 2014; Tsoras & Champion, 2018). A recent publication that used the PNP technologies developed by the Champion lab demonstrated a proof of concept of a universal influenza virus (Deng et al., 2018). NPs were comprised of a core of a tetramer of M2e epitopes from four influenza subtypes, and surface modified with one of two different recombinant mutants of the highly conserved hemagglutinin (HA) stalks from various subclasses of influenza. By creating a cocktail of two NPs, each modified with different recombinant HA variants, Deng et al. elicited universal protection to a wide variety of influenza subtypes. While the use of the highly conserved M2e epitopes has been attempted before in vaccines, these vaccines were constructed from virus-like particles (VLPs) loaded with epitopes and resulted in off target immune responses due to the carrier proteins in the VLP (Fiers et al., 2009; Turley et al., 2011). PNPs made almost entirely of proteins of interest, as

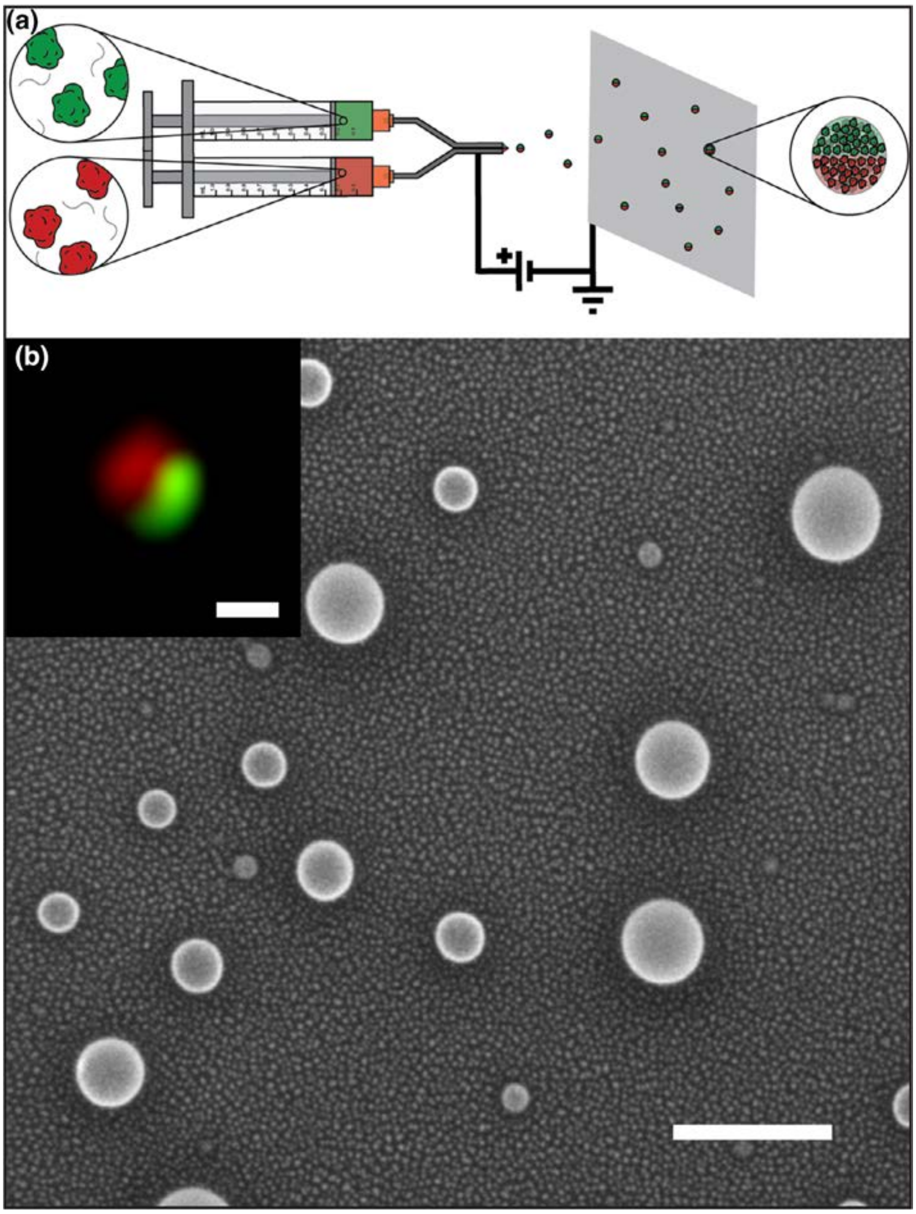

FIGURE 4 Multicompartmental protein nanoparticles can be made through electrohydrodynamic cojetting, as shown in (a). As an example, we synthesized particles with two compartments, one made of insulin and the other of hemoglobin. The particles are spherical as shown in the electron micrograph in (b), and show a clear bicompartmental nature when each compartment is selectively loaded with a fluorescent dye and imaged using structured illumination microscopy (insert). Scale bar is 200 nm

was demonstrated in the work by Deng et al., can avoid off target effect problems. Additionally, coacervation-manufactured PNPs, as opposed to the self-assembled or VLP counterparts, have greater stability over a large range of physiological environments, and studies have shown that they can potentially create cold chain-independent therapies (Chang, Deng, Wang, & Champion, 2018). A clear downside of coacervation particles is inherent in the simplicity of their synthetic method, in that it creates homogeneous distributions of proteins throughout each particle. Only radial complexity through surface modifications methods are able to provide any kind of anisotropy to the particles, as opposed to technologies such as those discussed in previous sections.

Another research avenue leveraging biologically-derived materials focuses on engineering extracellular vesicle-based systems such as exosomes. Their natural stability in the blood stream, presence of multiple adhesive proteins on their surface, and ability to transport functional biomolecules such as proteins and RNAs between cells, position them as potential drug carriers (Armstrong & Stevens, 2018; Batrakova & Kim, 2015; Van Dommelen et al., 2012). Exosomes have been exploited for targeted delivery of a wide range of therapeutic cargos such as siRNA to cross the BBB (Alvarez-Erviti et al., 2011), the chemotherapeutic drug doxorubicin to solid tumors (Tian et al., 2014) and the antioxidant agent curcumin to inflammatory cells (Sun et al., 2010). There are, however, limitations such as difficulties in scalability, and reproducibility, along with low drug loading capacity of well-characterized extracellular vesicles that have restricted their clinical and pharmaceutical adoption (Ingato, Uk, Jun, & Jik, 2016). In contrast with exosomes, particles that use proteins as their primary building blocks avoid most of these hurdles, and show similar positive characteristics. In addition, proteins have highly established manufacturing processes that have resulted in a plethora of products in the market, and thus have a more direct path to the clinic.

Knowledge of protein folding and biochemistry has advanced to the point where novel structures and functions can be built de novo through either first principles engineering, as demonstrated in a variety of self-assembled NPs previously described, or through directed evolution, as has been seminally shown by the work of Frances Arnold and colleagues (Arnold, 2018). However, the use of novel proteins does raise questions of translatability of technologies that are based on almost entirely recombinant proteins and have no analogues in the clinic or even the human body. Additionally, new proteins offer the ability to potentially use the function–follows–form principles of proteins to build complex, compartmentalized nanomachines from protein NPs, though this possibility has yet to be fully explored. The large number of different synthetic routes, functionalities and applications of NPs based on proteins as their building blocks that have been recently developed and commercialized shows the bright potential for PNPs as a revolutionary form of NPs in medicine.

5 CONCLUSION

The field of NP-based drug delivery systems is currently at the verge of entering the clinical arena. A wide range of clinical trials that involve nanomedicines are currently being pursued. Despite the increased acceptance and major technological progress in recent decades, the field of NP engineering is still severely limited by inefficient targeting and the lacking ability to cross challenging biological barriers, such as BBB. This fuels a continuous quest for novel and improved NP systems that can be accessed by scalable and flexible manufacturing processes. Building on previous work with EHD cojetting, novel polymer/protein hybrid NPs comprised of multiple internal compartments may become particularly attractive candidates for nanomedicine-based combination therapies (Figure 4a). In this case, polymer chemistry is merged with protein biochemistry to design NPs where proteins de facto become one of the repetition units in the synthetic biomaterial that constitutes engineered nanocarriers. Such an approach combines the versatility and targeting capabilities of synthetic NPs with the excellent circulation and biodistribution as well as rapid clearance in RES organs typically observed for proteins. As an example, scanning electron microscopy image of bicompartmental NPs made by EHD cojetting is shown in Figure 4b. Here, one compartment is made from a PEG/insulin “copolymer” system, whereas the second compartment is comprised of PEG/hemoglobin. Each compartment stained with a distinct fluorescent marker, and subsequently resolved using structured illumination microscopy is shown as an inset in Figure 4b. These and other advances will be necessary to design, engineer, and manufacture the next generation of NP drug delivery platforms.

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

AUTHOR CONTRIBUTIONS

Nahal Habibi: Visualization; writing-original draft; writing-review and editing. **Daniel Quevedo:** Visualization; writing-original draft; writing-review and editing. **Jason Gregory:** Visualization; writing-original draft; writing-review and editing. **Joerg Lahann:** Conceptualization; supervision; writing-review and editing.

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