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Nanoformulation properties, characterization, and behavior in complex biological matrices: Challenges and opportunities for brain-targeted drug delivery applications and enhanced translational potential

Vibhuti Agrahari [a], Pierre-Alain Burnouf [b], Thierry Burnouf [c,d,e,**], Vivek Agrahari [f,*]

[a] Bernard J. Dunn School of Pharmacy, Shenandoah University, Winchester, VA, USA
[b] Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan
[c] Graduate Institute of Biomedical Materials and Tissue Engineering, College of Biomedical Engineering, Taipei Medical University, Taipei, Taiwan
[d] International Ph.D. Program in Biomedical Engineering, College of Biomedical Engineering, Taipei Medical University, Taipei, Taiwan
[e] International Ph.D. Program in Cell Therapy and Regeneration Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan
[f] CONRAD, Eastern Virginia Medical School, Arlington, VA, USA

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ABSTRACT

Nanocarriers (synthetic/cell-based have attracted enormous interest for various therapeutic indications, including neurodegenerative disorders. A broader understanding of the impact of nanomedicines design is now required to enhance their translational potential. Nanoformulations *in vivo* journey is significantly affected by their physicochemical properties including the size, shape, hydrophobicity, elasticity, and surface charge/chemistry/morphology, which play a role as an interface with the biological environment. Understanding protein corona formation is crucial in characterizing nanocarriers and evaluating their interactions with biological systems. In this review, the types and properties of the brain-targeted nanocarriers are discussed. The biological factors and nanocarriers properties affecting their *in vivo* behavior are elaborated. The compositional description of cell culture and biological matrices, including proteins potentially relevant to protein corona built-up on nanoformulation especially for brain administration, is provided. Analytical techniques of characterizing nanocarriers in complex matrices, their advantages, limitations, and implementation challenges in industrial GMP environment are discussed. The uses of orthogonal complementary characterization approaches of nanocarriers are also covered.

Contents

* Corresponding author at: CONRAD, Eastern Virginia Medical School, 1911 North Fort Myer Drive, Suite 900, Arlington, VA 22209, USA.
** Corresponding author at: Graduate Institute of Biomedical Materials and Tissue Engineering, College of Biomedical Engineering, Taipei Medical University, 250 Wu-Shin Street, Taipei 11031, Taiwan.
E-mail addresses: thierry@tmu.edu.tw, thburnouf@gmail.com (T. Burnouf), vagrahari@conrad.org (V. Agrahari).

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1. Introduction

The current strategic arsenal available to treat human diseases, including neurological disorders, cancers, inflammatory and metabolic diseases, relies on the administration of chemical or biological drug formulations. Conventional therapies based on the administration of drugs often represent a sub-optimal clinical option associated with substantial clinical drawbacks occurring over the long-term administration periods that are required to address serious and complex pathologies. Systemic toxic effects are due to a lack of discrimination between normal and pathogenic tissues. The control and monitoring of toxicity pose critical difficulties in the management of patients, potentially requiring an interruption of treatment or a switch to an alternative, less effective, therapeutic approach. In addition, biological barriers, such as the blood-brain barrier (BBB), blood-retinal barrier, blood-labyrinth barrier, are largely impermeable to drugs, and therefore meaningful therapeutic drug doses cannot reach the pathological sites unless smart delivery approaches are developed [1–3]. Actually, it has been estimated in 2018 that 98% of currently available drugs are ineffective for the treatment of cerebral pathologies due to their inability to target the pathological sites [4].

For brain diseases, the majority of the therapeutic molecules do not readily permeate into the brain parenchyma, which is one of the most significant challenges of an effective central nervous system (CNS) drug delivery. This is mainly due to the presence of the BBB. Transport of substances across the BBB is selective and limited due to the presence of tight junctions (physical barrier) and metabolic barriers (enzymes and transport systems) [5]. BBB separates the brain extracellular fluid from the blood, transport endogenous and essential small molecular mass nutrients into, and filter harmful compounds out of the brain to the systemic circulation. The specific and selective permeability of BBB for various components is due to special features of the brain cells including the excess metabolic capability of BBB tissue, the fact that brain capillary endothelial cells are 50-100 times tighter than the normal circulation endothelial cells, and the trans-endothelial electrical resistance of the junctional complex that fuses the brain capillary endothelial cells that is higher than that of most non-cerebral capillaries. In addition, the expression of ion channels and the influx/efflux transporters at the BBB sites restrict the permeability of drugs and other molecules [5]. An effective crossing of the BBB can only be obtained by molecules that are, among other criteria, less than 400 Da (daltons), lipid-soluble, and less than 8-10 hydrogen bonds; this notably excludes most pharmaceuticals and biologicals from entering the brain at therapeutically effective levels [6]. Local reversible disruption of the BBB using microbubble-facilitated focused ultrasound is so far limited to very specific situations, as is the case for the delivery of chemotherapeutic agents [7]. Hence, alternative drug delivery approaches have been used to overcome the transport limits of BBB and one of the most promising ones is the use of nanocarriers [1,8–11]. However, nanoformulations when introduced into complex biological media, get exposed to biomacromolecules, proteins, etc., which modify their intrinsic properties and *in vivo* behavior, significantly affected by various physicochemical and biological factors. Hence, analyzing nanocarriers behavior and interactions in biological fluids is vital.

In this review, various types of brain-targeted nanocarrier systems are briefly discussed. The biological factors and nanocarriers physicochemical properties affecting their *in vivo* journey are elaborated. The composition of cell culture media and *in vivo* biological matrices are discussed. The presence of several proteins in these complex matrices, potentially relevant to protein corona formation on nanocarriers is

provided including the discussion on biological, *in vitro*, and nanocarrier formulation factors affecting the protein corona and protein adsorption. Analytical techniques of characterizing nanocarriers' physicochemical properties in complex matrices including the applicability and challenges in implementing these analytical methods in the industrial environment are summarized. Moreover, the advantages of using complementary characterization approaches of nanocarriers' properties and protein corona formation are also discussed. The overall objective here is to generate a broader understanding of the properties and the impact of brain-targeted nanomedicines optimal design with the hope that these early stages learning and considerations will help in enhancing nanomedicine products translational and therapeutic potential for CNS diseases. The nanoformulation(s), nanocarrier(s), and nanomedicine(s) terms are interchangeably used, but have the similar meanings in the context of the discussions in this manuscript.

2. Synthetic nanocarriers for neurodegenerative diseases

Liposomes have been the first nanoformulation to be licensed for clinical use; PEGylated liposomal doxorubicin has been approved for the treatment of AIDS-related Kaposi's sarcoma, ovarian cancer, and lymphoma. NP albumin-bound paclitaxel is used against breast cancer and non-small cell lung cancer [12]. In general nanocarriers are nanoscaled systems, capable of encapsulating both small drugs as well as macromolecules. These colloidal formulations provide flexibility in modifying their physicochemical properties and surface chemistry/functionality that ultimately help in enhancing the therapeutic efficacy and bioavailability of drugs across the biological barriers such as BBB [13]. The purpose of drug nanocarriers can be manifold and encompasses for (a) drug stabilization, (b) reduction in toxic effects, (c) capacity to cross biological barriers, and (d) improved targeting to the disease site [12]. Biomedical engineers have come up with an impressive range of experimental synthetic drug delivery system (DDS) platforms for nanomedicine applications, as illustrated in Fig. 1. These platforms include lipid-based nanocarriers (e.g. liposomes), polymer-based nanocarriers (e.g. micelles, nanoparticles: NPs, NP albumin-bound technology), inorganic NPs (silica NPs, metal NP, hafnium oxide NPs), drug conjugates (e.g. antibody-drug conjugate), nanocrystals, and viral NPs [14–16].

There is tremendous interest and need in designing novel nanotherapeutic platforms capable to encapsulate, or to complex, drugs as a means to improve their efficacy and safety, while circumventing the drawbacks of conventional drug therapy [17,18]. The specific attributes of nanoformulation intended for brain delivery and treatment of neurodegenerative diseases should encompass lack of toxicity, efficient diffusion within the brain regions, size ranging from a few to ca. 200 nm, absence of neuro-inflammatory effects, targeting ability, efficacy, and affordability. The properties that govern the characteristics of the nanocarriers are their size, surface charge, shape, morphology, and surface functionality. We briefly discuss below several types of nanocarrier systems used for targeted drug delivery applications to the brain.

Polymeric NPs are colloidal systems, usually synthesized from biodegradable polymers, such as poly(lactic-*co*-glycolic acid) (PLGA), in the size range of 10-1000 nm. These systems offer the potentials of controlling the drug release profile to provide the long-term sustained or burst drug release, as needed. Depending on the specific method of preparation, polymeric NPs can be nanospheres or nanocapsules. Polymeric NPs also provide the flexibility of surface modification by targeting molecules, encapsulation of multiple drugs, adjustable size, shape, and surface charge. Hence, these systems have been extensively used in neurodegenerative diseases and brain cancer [19–22]. However, the short circulation time and difficulty in uniform and robust manufacturing of polymeric nanoformulation are their major limitations.

Liposomes are vesicles made of phospholipid bilayers enclosing an aqueous core. The core can be encapsulated with hydrophilic drugs whereas the lipid bilayer can be loaded with the hydrophobic drugs. Changing the lipids ratio, types and the manufacturing process can modify the physiochemical properties of liposomes. The surface of liposomes can be functionalized to make them long-circulating and targeted systems. Liposomes have been comprehensively used in the targeting of brain diseases owing to their ability to cross the BBB [23–25]. Moreover, due to the site-specific targeting properties of the liposomes, dual targeted liposomes (receptor-mediated and/or adsorption-mediated) have recently been evaluated [26–28].

Nanomicelles are self-assembled systems made from biodegradable amphiphilic block polymers. They can encapsulate hydrophobic drugs in the core and their hydrophilic shell allows the encapsulation of hydrophilic drugs. Moreover, the hydrophilic shell provides stability and long circulation time to the nanomicelles [29,30]. Due to several advantages provided by nanomicelles including the targeting ability, these formulations are widely used in developing the brain-targeted delivery systems [31–34]. Nanomicelles can also be modified to provide a stimuli-responsive drug release formulation. Recently, wormlike nanomicelles are developed to penetrate deep into the brain tumor and inhibit its progression. These micelles possess unique pH-responsive properties and can be dissociated at intracellular acidic environments to release the therapeutic agent [35].

Nanoemulsions composed of either water in oil (W/O) or oil in water (O/W) droplets, stabilized by an amphiphilic surfactant, represent a promising delivery system for encapsulation of drugs and their protection from degradation, controlling their release and enhancing their bioavailability [36]. Based on these attributes, nanoemulsions have been used extensively for brain delivery especially through the intranasal route of administration, which is a non-invasive delivery option bypassing the BBB and allowing direct access of various biologics-based drugs to the CNS [37–40].

Dendrimers are branched synthetic polymeric macromolecules in the nanoscale size range. Various types of dendrimers based on polyamidoamine, polypropylenemine, and poly-L-lysine have been explored as drug delivery vehicles [41,42]. The specific advantages of dendrimers include their uniform size distribution, availability of multiple locations for drug and ligand conjugation, conjugation of multiple molecules at the same time, and high thermodynamic stability [41,42]. Use of dendrimers for brain diseases has been explored significantly in recent years and showed beneficial responses [43–49]. However, the complexity of their development and toxicity issues limits their applicability.

Inorganic NPs based on gold, silica, carbon, and iron are widely used in brain-targeted drug delivery applications, as they possess unique characteristics. These systems can be easily modified to facilitate conjugation of ligands or polymers and it is possible to tune their size and shape for specific applications. Inorganic NPs has the ability to permeate through the brain microvasculature due to their small size and the large surface area allows them to be coated with a variety of ligands for targeting approaches. Thus, these systems are explored in brain targeting approaches [50–54]. Magnetic NPs are commonly comprised of core-shell morphology with an iron oxide core coated with a biocompatible material. They can be systemically administered into the Bloodstream and targeted by applying an external magnetic field for the therapeutic, imaging or diagnostic applications [55–58]. Furthermore, external or internal stimuli can control the drug release from inorganic nanocarriers across BBB and facilitate on-demand delivery [59,60]. Despite the fact that inorganic NPs possess many unique advantages, their non-degradability, toxicity, and *in vivo* clearance remain limiting factors in their application for the treatment of neurodegenerative and other diseases.

2.1. Therapeutic limits and specific challenges in the design of nanocarriers

Clinical experience shows that these nanocarrier-based drug delivery approaches do decrease the risks of drug resistance and improve

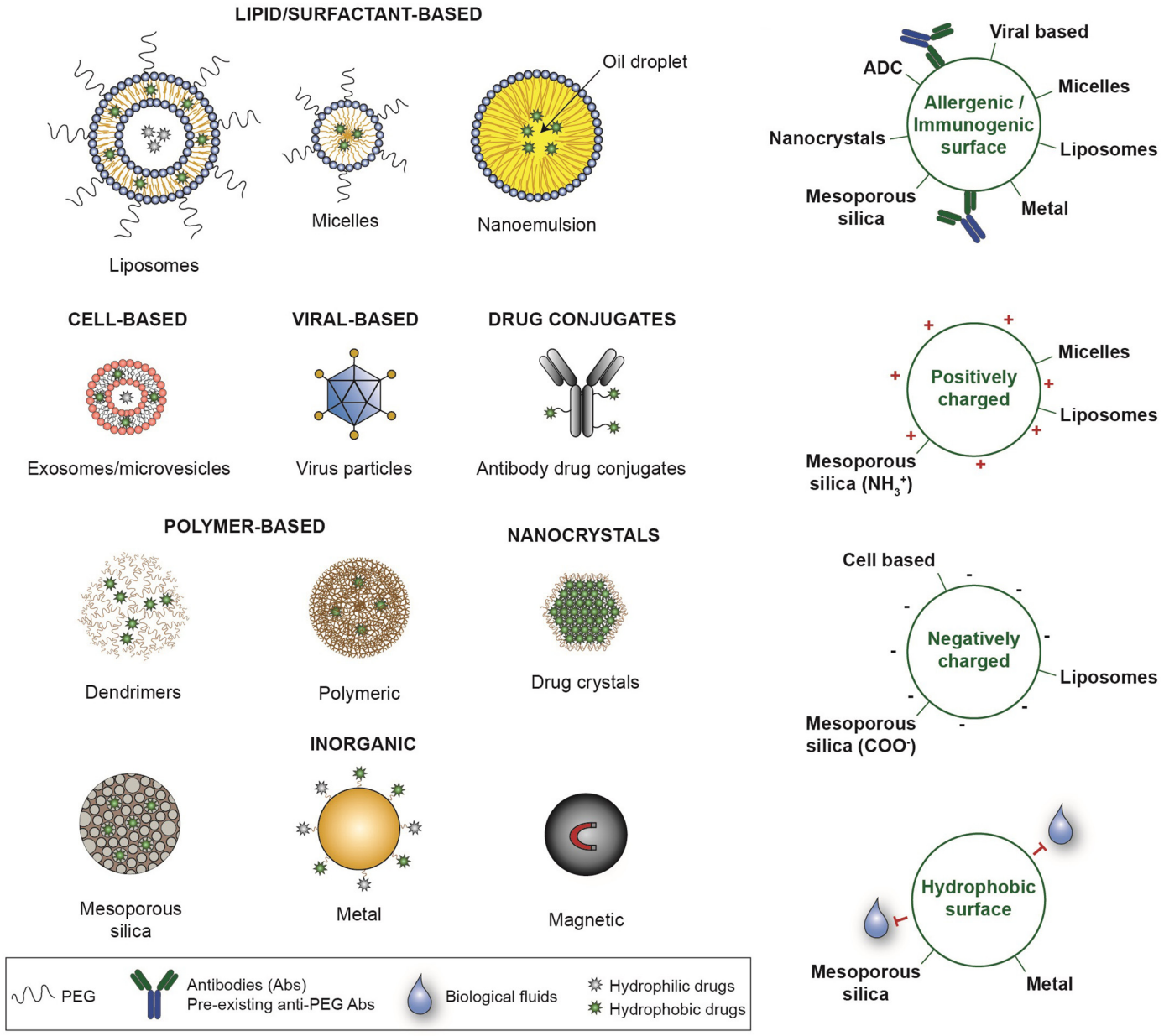

Fig. 1. Schematic illustration of various types of nanocarrier systems.

drugs solubility and stability, making drug formulation more straightforward and less toxic [61]. Still, they do not prevent all side-effects as, for instance, 10-15% of patients receiving liposomal doxorubicin get skin reactions, peripheral neuropathy, and hypersensitivity reactions [12]. Complexation with PEG itself, to improve the blood circulation of nanocarriers, does not result in an immunologically inert chemical entity, as many patients have pre-existing anti-PEG antibodies, a physiological situation that is suspected to decrease drug efficacy and tolerance, in particular by affecting the liposome outer structure and protein corona [62–64]. In addition, specific types of nanocarriers have their own limitations such as complex manufacturing process, low drug encapsulation efficiency, instability issues, and safety concerns. Another significant issue is the lack of targeting potential. Indeed, drug delivery towards, and diffusion within the pathological site, such as a tumor, is presumed to rely only on the enhanced permeation and retention (EPR) dogma, a biophysical effect, which was experimentally evidenced in rodents, but is actually uncertain to occur in humans or at least is not universal [65,66]. In cancer pathologies, it has been estimated that only a mean 0.7% of drugs loaded into nanocarriers reaches a solid tumor [67], leading to treating patients with massive doses to yield therapeutic effects, detrimentally impacting treatment safety and cost. Another limit of synthetic "undecorated" NPs/nanocarriers is their poor capacity to overcome cellular barriers prior to releasing the encapsulated therapeutics [68].

Nanoformulation intended for brain delivery pose specific safety issues that need to be addressed carefully, as the brain is a particularly delicate organ susceptible to toxic substances [69]. Other challenges include their limited access to the CNS due to the BBB barrier, the limited diffusion through the brain parenchyma, the risks of inducing neurotoxicity, pathological changes in gene expression, neuroinflammation, and limited targeting [70]. Brain targeting by nanoformulation to treat neurodegenerative disorders and brain injuries (such as traumatic brain injury, ischemic or hemorrhagic strokes) needs design or administration strategies capable to by-pass the BBB and/or the blood -cerebrospinal fluid barrier (BCSFB), which both preclude passive diffusion into the brain. Approaches to enhance drug delivery to the brain include invasive methods by intracerebroventricular, intracerebral or intrathecal administration [71–73], and non-invasive methods like intranasal

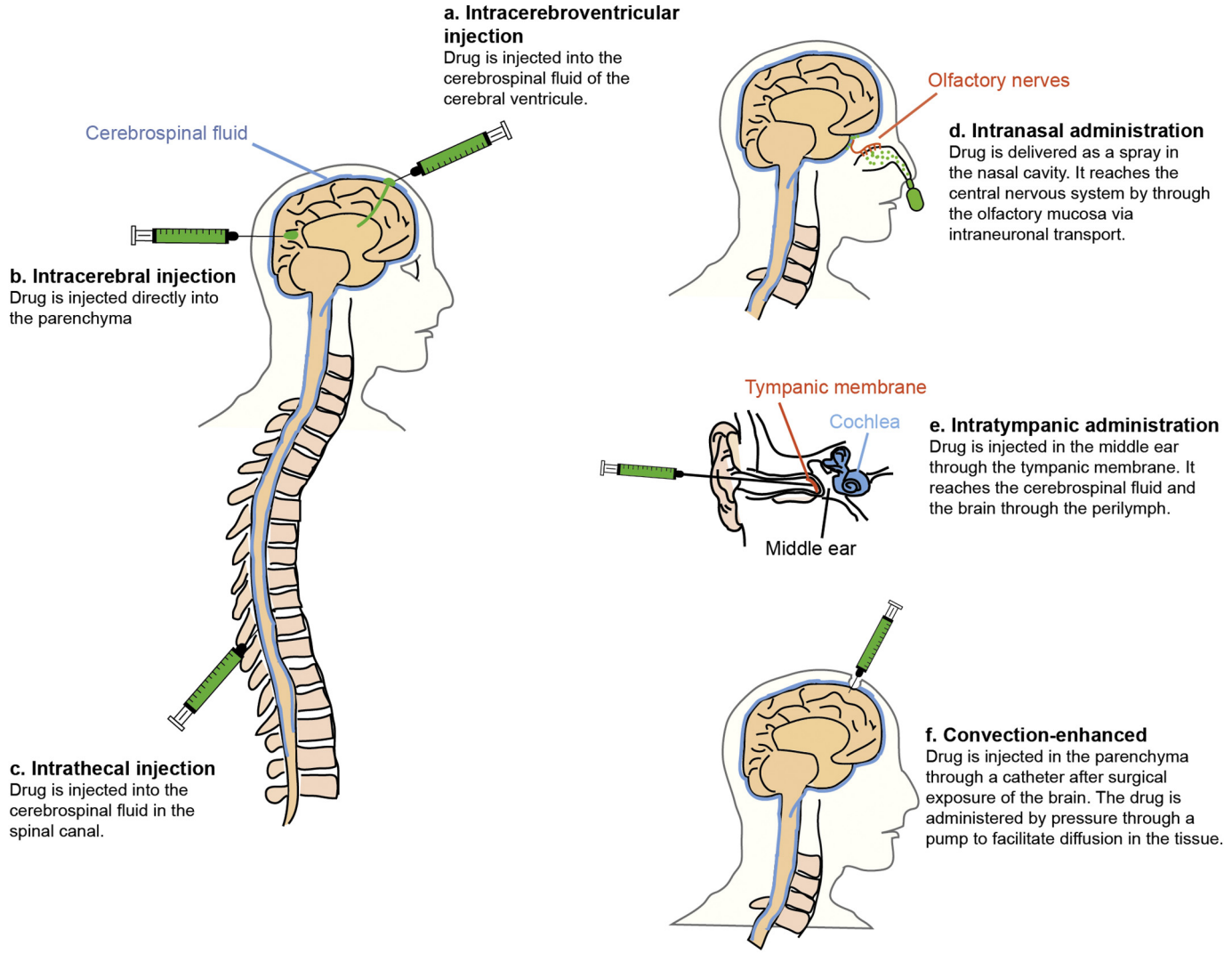

Fig. 2. Summary of drug delivery methods (noninvasive and invasive) that bypass the blood-brain barrier (BBB).

administration [74], a route that exploits the capacity of the olfactory and trigeminal neuronal pathways to by-pass the BBB, intratympanic administration [75], or convection-enhanced delivery [76] a mode of administration considered in the treatment of brain tumors but also neurodegenerative diseases. Intravenous administration of DDS may not be optimal to ensure brain tissues targeting. Some strategies may combine intravenous administration with attempts to induce transient disruption of the BBB by various approaches involving the use of hyperosmotic or vasoactive substances [77], and, more recently, magnetic resonance-guided focused ultrasonic irradiation combined with microbubbles [78,79]. Fig. 2 provides a schematic summary of various strategies and administration routes that can be used to deliver therapeutics to the brain.

Limits in targeting and biological barriers permeability may potentially be overcome however by new drug administration routes and delivery strategies using biologically-inspired nanocarriers mimicking the physiological targeting capacity provided by cell membranes [80] or even by using cells, such as red blood cells (RBCs), or cell-derived nano-microvesicles themselves as enzymatic bioreactors or DDS [14,16,67,81,82]. In that regards, it is likely that research work is going to intensify in the development of nanoformulations dedicated to the administration of drugs by the non-invasive nose to brain delivery route, as it can avoid the uncertainties associated with the impact of the blood compartment [4]. Although uncertainties still persist with regards to its effectiveness, many experimental studies concur to consider the intranasal route as providing rapid (less than one hour) delivery of some drugs to the CNS [4,83]. In order to avoid potential degradation of the therapeutic compounds as well as potentially optimize their delivery and diffusion in the brain, nanoformulation systems may be of primary interest. For instance, lipid-based NPs of a 50-1000 nm range have been shown of specific superiority for this purpose [4,84].

3. Cell-based and biologically-inspired nanoformulation

Whole cells, including Mesenchymal stem cells (MSCs), RBCs, platelets, monocytes and macrophages are increasingly considered as potential drug carrier options [85,86] due to several advantages that include their immune transparence, high loading capacity, non-toxicity, long residence time, targeting ability provided by membrane receptors, and, for some of them, tissue infiltration capability [81,86]. Under normal physiological conditions, the BBB protects the brain against the passage of most intact cells, therefore limiting the possibility of relying on intravenous administration of cells or cell-based carriers, and leading researchers to evaluate by-passing strategies. Most studies so far have focused on using naïve or modified progenitor cells or MSCs as potential

therapies of neurological disorders thanks to their unique capacity among cells to cross the BBB [85,87], and their expected capacity to be able to home in the brain, as long as they do not lead to an allogeneic inflammatory response, and if their potential differentiation into specialized cells is controlled [88]. The activity of naïve MSCs in tissue repair is thought to be due to complementary factors, including the release of a range of trophic factors among cytokines and growth factors that induce immunomodulatory and anti-inflammatory actions. MSCs have been used as a carrier for carboxylesterase, suicide genes, or miRNA, trying to benefit of the propensity of MSC to home at the pathological glioma site [88]. There are still relatively few studies using MSCs as a carrier for proteins, drugs, gene or RNA for treatment of brain diseases.

Several approaches have been evaluated in the field of neurodegenerative diseases. *Ex vivo* expanded human neural progenitor cells (hNPC), modified using lentivirus to express glial cell line-derived neurotrophic factor (GDNF), have been transplanted into the lumbar spinal cord of rats overexpressing the G93A SOD1 mutation ($SOD1^{G93A}$), as model for amyotrophic lateral sclerosis (ALS), in a therapeutic strategy for glial cell replacement and trophic factor delivery [89] Genetic modifications of MSC to secrete IFN-β, could counterbalance the effects of pro-inflammatory cytokines upon intravenous administration in a mice model of multiple sclerosis [90]. Experiments have shown that MSCs, potentially pre-treated by hyaluronidase, have the capacity to penetrate the brain by the intranasal route using two pathways by migrating into the olfactory bulb towards the brain, and entry into the cerebrospinal fluid (CSF) and the brain parenchyma [91]. Due to several factors, including potential risks of immunogenicity and tumorigenicity associated with MSCs, researchers are increasingly focusing on the use of extracellular vesicles (EVs), including exosomes and microvesicles, as alternative therapeutic strategies and drug carriers to the brain [92,93].

Exosomes are nano-sized EVs with a bilayer lipid membrane and an aqueous core. They are naturally produced in body cells and can be found in several body fluids, including blood, saliva, urine, and CSF [94]. The capacity of exosomes and other EVs to encapsulate hydrophilic/hydrophobic molecules, nucleic acids, proteins, lipids, and their role in intercellular communication and capacity to overcome tissue barriers make them a versatile platform for drug delivery applications [95], although substantial challenges must still be overcome for their widespread clinical translation [96]. Exosomes have many other desirable features such as long circulating half-life, intrinsic ability to target tissues, higher biocompatibility, and their ability to cross tissue barriers including the BBB [92,97]. Blood exosomes were recently used as a delivery system of dopamine and found to have the capacity to cross the BBB and to reach the striatum and the substantia nigra in an animal model of Parkinson's disease (PD), with improved efficacy and lower toxicity than free dopamine [98]. Similarly, naïve exosomes from macrophage were shown experimentally to be able, upon intravenous administration, to penetrate the BBB through several mechanistic interactions with brain microvessel endothelial cells of the BBB and to deliver BDNF [99]. This capacity was also found under inflammatory conditions typical of neurodegenerative disorders, opening perspectives in the use of such exosomes as nanocarriers to treat CNS diseases [99]. A study confirmed that brain endothelial cell-derived exosomes can deliver anticancer drugs across the BBB for the treatment of brain cancer in a zebrafish (Danio rerio) model [100]. Exosomes may also emerge as an interesting candidate for intranasal drug delivery. Monocytes and macrophages-derived exosomes have been used as vectors to incorporate catalase as antioxidant to treat PD. Their intranasal administration was found to exhibit neuroprotective effects in an *in vivo* PD model [101]. These prominent abilities of exosomes have been exploited into several different applications [102]. However, the isolation, characterization, large-scale production, and purification of exosomes, and other extracellular vesicles, are major challenges and needs further understanding and development [100,103].

4. Physicochemical properties of brain-targeted nanocarrier systems: importance in drug delivery applications and biological behavior

As discussed earlier, different types of nanocarriers have been used for efficient delivery of therapeutics so that they can reach their target sites in the brain. In these systems, several of their characteristics such as the surface charge, size, shape, morphology were engineered to provide designed functionalities to enhance their brain permeability and bioavailability of encapsulated therapeutics [104]. However, nanoformulation *in vivo* journey is significantly affected by several variables (Fig. 3). The primary features and characteristics of nanoformulations influencing brain delivery and BBB transport are briefly discussed here and summarized in Fig. 4.

4.1. Size

Particle size is one of the most critical parameters in nanocarriers design that significantly influences the cellular uptake, biodistribution, and clearance [105,106]. Several studies have investigated the effect of NPs size on the probability to cross the BBB and into the brain. Barbiturate coated gold NPs (GNPs) were synthesized in 20, 50, 70 and 110 nm size. Results showed that the intracellular uptake of GNPs was size dependent, 70 nm being optimal for the maximum amount of gold within the brain cells, and 20 nm for the maximum free surface area [107]. The correlation among silica NP size (30, 100, and 400 nm) and BBB penetration was confirmed using *in vitro* model. The permeability of NPs was determined using the permeability coefficient (P(app)). Results revealed that the Papp for the 30 nm NPs was higher than that of larger NPs [108].

Recently, PLGA-PEG NPs of 100 nm exhibited deeper brain penetration and longer circulating time than that of the 200 and 800 nm NPs when administered intravenously [109]. Furthermore, one study used 10, 30, and 60 nm biocompatible near-infrared NP; the 10 nm NPs showed poor selectivity, the 30 nm NPs were the most sensitive and selective for BBB damage evaluation, and the 60 nm NPs barely crossed the BBB [110]. Both the 10 and 30 nm NPs crossed the BBB, however, the 10 nm NPs penetrated and leaked from the nonischemic region of the brain. Additionally, 60 nm particles couldn't cross the damaged BBB due to the bigger size. As such, 30 nm particles showed a superior capability for BBB damage evaluation. Although smaller sized NPs were transported through the BBB more easily, they led to limited encapsulation efficiency and rapid drug release [110].

The effect of NP size on blood-brain tumor barrier (BBTB) permeation of fluorescence-labeled gold NPs was investigated in a mouse model of orthotopic glioblastoma multiforme (GBM). The smallest 10 nm NPs were widely distributed in the brain tumor tissue, whereas the 50 and 100 nm NPs were located near the blood vessels [111]. Transport of PEGylated silica NPs with 100, 50, and 25 nm size across the BBB was evaluated using an *in vitro* BBB model. The BBB transport efficiency of NPs was found to be size-dependent, with increased particle size resulting in decreased efficiency [112]. The effect of insulin-coated GNPs (20, 50 and 70 nm) on their ability to cross the BBB was quantitatively investigated in mice. The insulin coating of the particles enabled targeting of specific brain regions and the biodistribution and highest accumulation within the brain was observed with 20 nm GNPs, 2 h post injection [113].

The above few examples clearly indicated that particle size is an extremely important parameter that has to be evaluated early while designing brain-targeted nanocarrier systems. In general, the smaller (< 200 nm) the particle size of NPs is, the better is the efficiency in crossing the BBB, but, this needs to be evaluated on a case-by-case basis as size has an impact on the drug loading and drug release profile of nanocarriers and needs to be optimized accordingly.

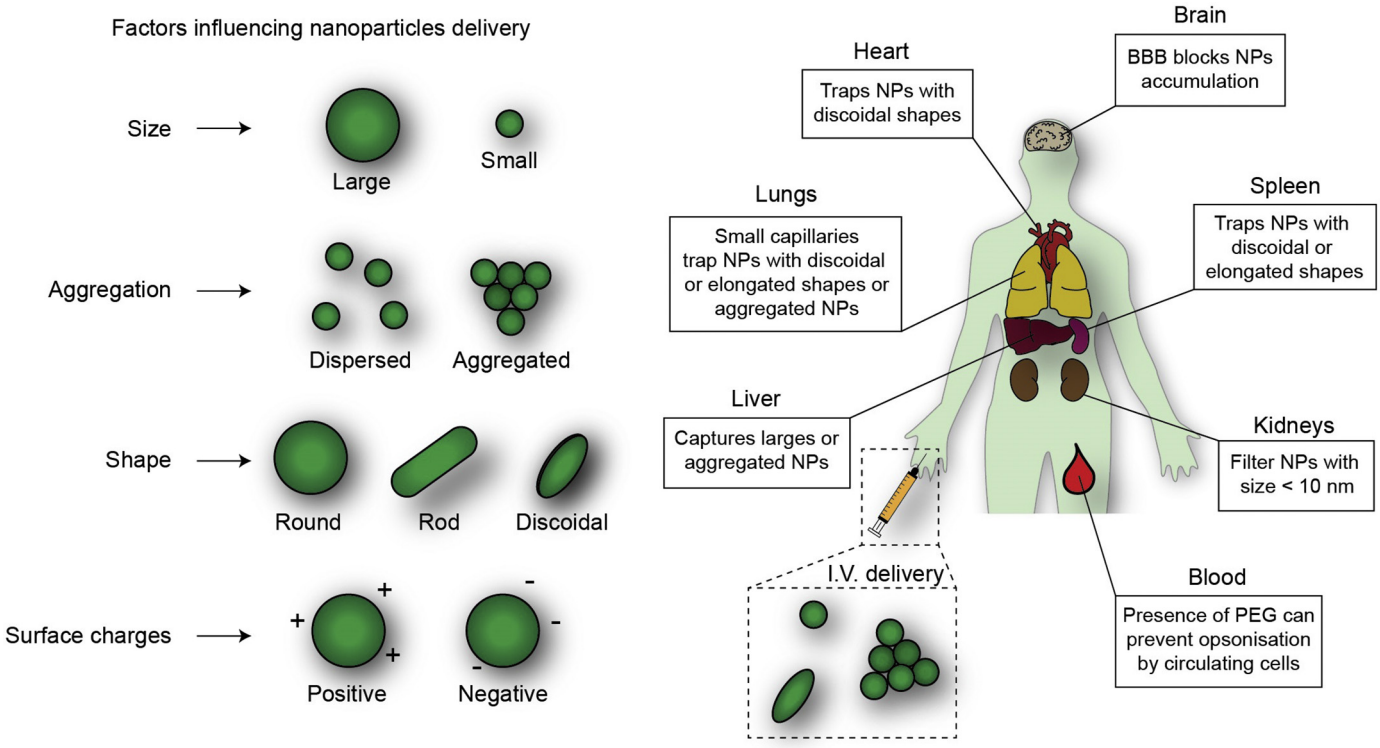

Fig. 3. Schematics of nanoformulation *in vivo* journey primarily mediated by particle size, shape, and surface charge.

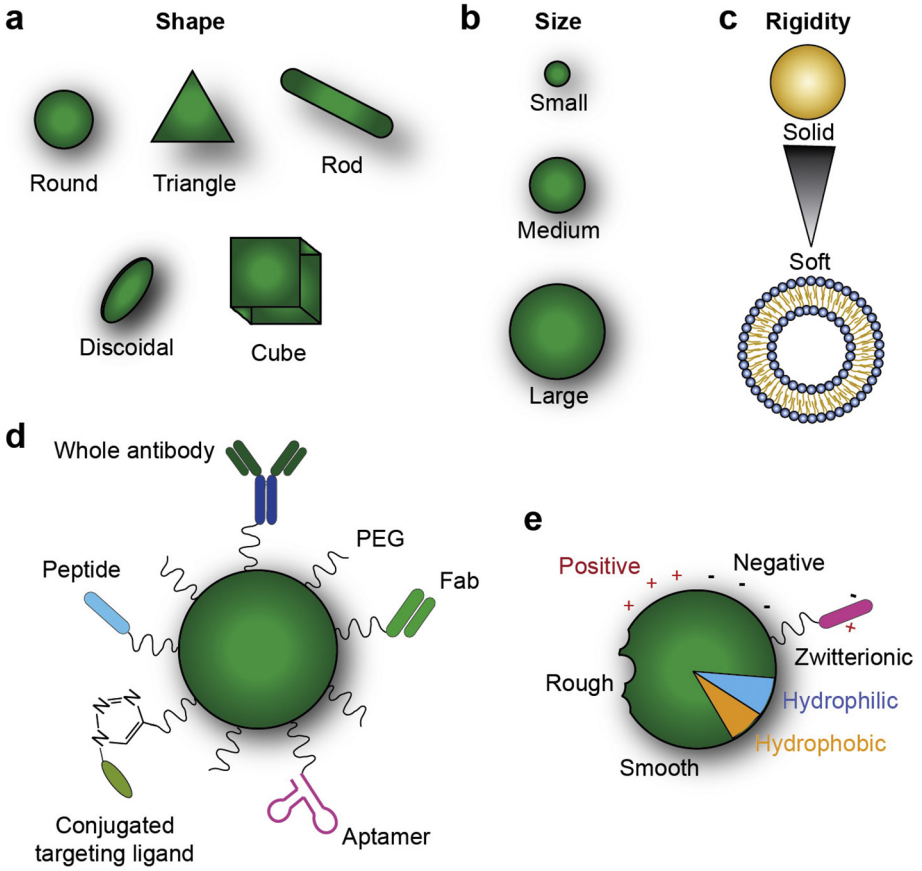

Fig. 4. Nanocarrier physical and chemical properties influencing systemic delivery and blood-brain barrier (BBB) passage. (a) Shape. (b) Size. (c) Rigidity. (d) Surface functionality. (e) Surface charge.

4.2. Shape

The shape of nanoformulations is an important parameter that affects their biological performance such as biodistribution, circulation stability, cell uptake, and targeting ability to the brain [114,115] including the transport properties of NPs, such as particle velocity, diffusion, and adhesion to blood vessels walls, and interaction with cells [116]. Moreover, for targeting ability of NPs, not only the size and overall surface area, but also the shape, affects NPs receptor interaction and proteins adsorption. Bartczak *et al.* studied GNPs of four different shapes: spherical, rod-shaped, hollow, and silica-gold core-shell particles. Results showed that the cellular uptake of particles of different shapes was different and the uptake of spherical particles was the highest and that of hollow particles the lowest [117]. *In vitro* studies have demonstrated that rod-shaped polystyrene NPs ($501 \pm 43.6 \times 123.6 \pm 13.3$ nm) coated with an antibody against the transferrin receptor showed a 7-fold increase accumulation in brain endothelium compared to the spherical NPs of size ~200 nm [118].

Mesoporous silica NPs (MSNs) of different morphology and surface characteristics were investigated for drug carriers to the brain using *in vitro* BBB model [119]. The spherical and rod-shaped MSNs (bare MSNs and MSNs coated with a PEG-PEI block copolymer) in the sub-100 nm range were studied. There was more efficient and robust uptake of copolymer-coated NPs compared to uncoated particles. While the shape effect was detectable but small, coating with PEG-PEI copolymers facilitated the uptake of MSNs. Microscopy evaluation confirmed the intracellular presence of particles of both shapes [119]. The following two studies also showed that because nanocarrier shape was probably changed, they had effectively crossed the BBB. For example, flexible polymeric nanofiber implants were generated for prolonged and sustained release of an anti-glioma drug. PLGA, PCL and PLA polymers were used to create a library of nanofiber blends with *in vivo* drug release kinetics in brain varying from hours to months. Orthotopic rat glioma implanted wafers showed constant drug release (116.6 μg/day) with negligible leakage into the peripheral blood (<100 ng) rendering ~1000 fold differential drug dosage in tumor versus peripheral blood. Moreover, by combining different fibers into a single implant, controlled drug release for an extended duration of up to 30 days was achieved [120]. The concept of employing an amphiphilic derivative of a peptide to deliver the peptide into the brain was also demonstrated. The amphiphilic peptide was designed to self-assemble into nanofibers, wherein the active peptide epitope was tightly wrapped around the nanofiber core. The nanofiber form appeared to protect the amphiphilic peptide from degradation while in the plasma, and the amphiphilic nature of the peptide promoted its transport across the BBB. Therapeutic brain level of the amphiphilic peptide was achieved compared to when the non-derivatized peptide was administered [121].

Although methods such as mechanical stretching, self-assembly, soft lithography, microfluidics-based, template-assisted self-assembly have been recently developed for fabricating particles of varied shapes and sizes [122], the detailed impact of nanoformulation shape has not been fully elucidated for brain-targeted drug delivery applications, mostly due to the lack of manufacturing methods with appropriate control on the particle shape.

4.3. Elasticity

Nanocarrier flexibility or elasticity contributes to their targeting, endocytosis, and phagocytosis processes. Softer particles have a prolonged blood circulation time and increased organ deposition due to the deformation of softer particles by macrophages into shapes which are more difficult to internalize [123]. Recent studies confirmed that particle elasticity significantly alters the NPs cell uptake, circulation, targeting ability, and biodistribution [123–126]. Moreover, advances in particle synthesis have facilitated research into the role that particle elasticity plays in modulating the drug delivery efficacy of various systems [127]. PEG-based hydrogel NPs of 200 nm size and with elastic moduli ranging from 0.255 to 3000 kPa have been synthesized to investigate the role of particle elasticity on blood circulation time, biodistribution, targeting, endocytosis, and phagocytosis. Results demonstrated that softer NPs (10 kPa) had enhanced *in vivo* circulation and targeting compared to the harder NPs (3000 kPa). Furthermore, *in vitro* experiments showed that softer NPs exhibited significantly reduced cellular uptake in immune, endothelial, and cancer cells [123]. The *in vitro* cellular and *in vivo* tumor uptakes of nanolipogels (NLGs) composed of identical lipid bilayers encapsulating an alginate core, with tunable elasticity were evaluated. The elasticity of NLGs ranged from 45 ± 9 to $19{,}000 \pm 5$ kPa. Neoplastic and non-neoplastic cells exhibited significantly greater uptake of soft NLGs (Young's modulus <1.6 MPa) compared to their elastic counterparts (Young's modulus >13.8 MPa). Moreover, soft NLGs accumulated significantly more in tumors, whereas elastic NLGs preferentially accumulated in liver [128].

As like the particle shape, the impact of elasticity of nanoformulations on their biological roles, including the BBB transport has not been investigated much, primarily due to challenges in measuring and tuning NPs elasticity while maintaining their original size, shape, and surface chemistry [114]. An important work from Anselmo *et al.* [123] demonstrated that softer NPs offered enhanced *in vivo* circulation and targeting compared to harder NPs, including significantly reduced cellular uptake in immune cells *in vitro*. These observations suggested that nanoformulation elasticity is an important factor to be considered in improving the therapeutic potential of brain-targeted nanocarriers. Although not much has been done to determine the role of nanoformulation elasticity in brain-targeted drug delivery approaches, the above few studies unveiled that elasticity is an important parameter to be evaluated in determining NPs ability to cross the BBB.

4.4. Surface charge

Surface charge affects the biological performances such as solubility, clearance, biodistribution, stability, cell uptake, tissue diffusion, and cytotoxicity of NPs [129,130]. In general, the positively charged particles are taken-up more easily by cells than negatively charged or neutral particles because of the negatively charged nature of the cell membrane. The presence of glycoproteins and glycolipids creates a net negative charge on BBB; hence, negatively charged particles are electrostatically repelled from crossing the BBB and can enter the brain cells only through transport or receptor-mediated endocytic process. Thus, cationic NPs tend to achieve a higher CNS drug concentration [131,132]. However, positively charged NPs are rapidly eliminated from *in vivo* systems [132]. Moreover, NPs with neutral and negative charges decrease the adsorption of serum proteins, resulting in longer blood circulation times [133]. In addition, a positive charge on NPs surface could also result in non-targeted distribution as all the body cells are negatively charged. Results also showed that neutral NPs and low concentrations of negatively charged NPs have no effect on BBB integrity, whereas the BBB was disrupted in the presence of positively charged and high concentrations of negatively charged NPs [131,134]. A few examples below are discussing the effect of surface charge in brain-targeted DDS.

Surface modifications of ultra-small nanostructured lipid carriers (usNLC) with a particle size of ~44 nm were performed via the introduction of a positive charge to site-specific delivery for GBM treatment [135]. Monomeric and gemini surfactants, either with conventional head groups or serine-based ones, were tested for the surface modification, and the respective safety and efficacy to target GBM was evaluated. Monomeric serine-derived surfactants displayed the best performance, considering altogether particle size, zeta potential, cytotoxic profile, and cell uptake. Although conventional surfactants were able to produce usNLC with suitable physicochemical properties and cell uptake, they had high cytotoxicity [135]. Dual-targeting paclitaxel-loaded NPs were developed by decoration with negatively charged peptide-22 (with a

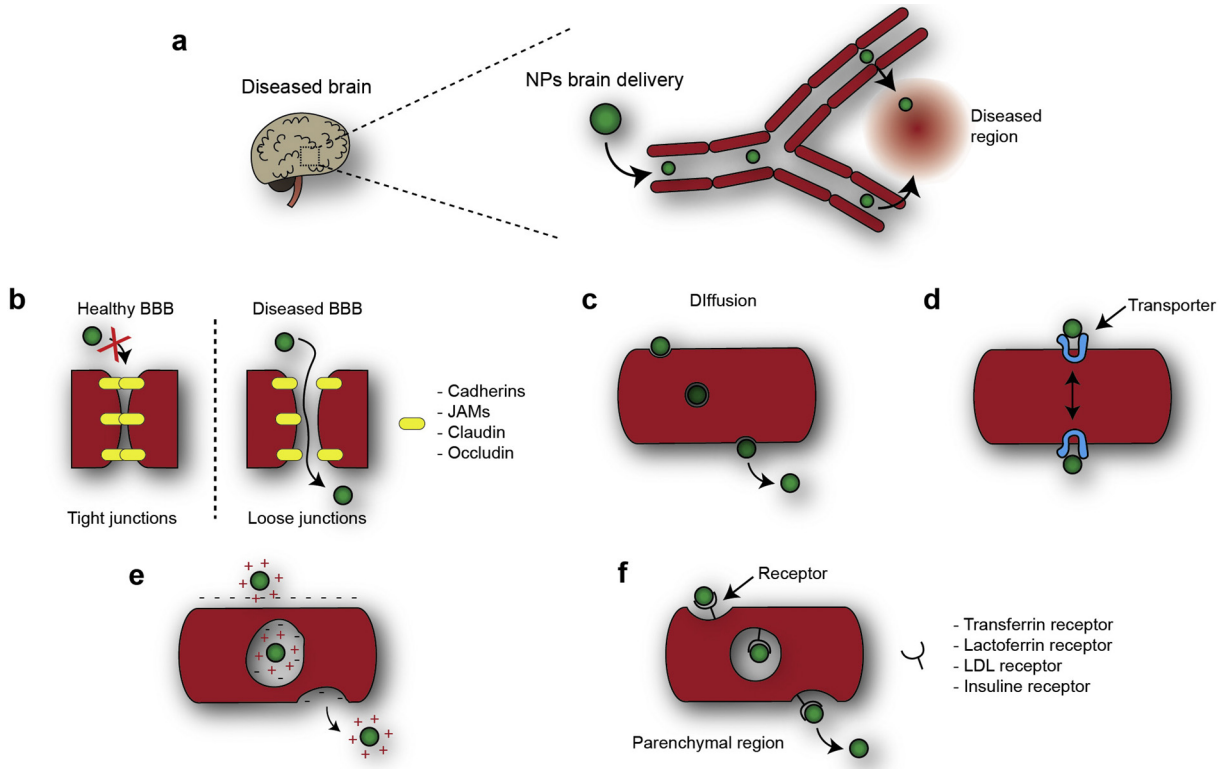

Fig. 5. Brain-targeted drug-delivery strategies through paracellular transport, passive transcellular diffusion, carrier-mediated transport, adsorptive mediated transcytosis, and receptor-mediated transcytosis.

special affinity for low-density lipoprotein receptor) to transport the drug across the BBB and then target brain tumor cells. Results demonstrated that peptide-22-decoration significantly increased the cellular uptake of NPs by C6 cells and BCECs but not by H92c(2-1) cells. *In vitro* BBB model showed that peptide-22 decorated NPs significantly increased the transport ratio of paclitaxel across the BBB and induced the apoptosis of C6 glioma cells below the BBB [136].

The movement of NPs of various diameters and surface coatings within fresh human and rat brain tissue *ex vivo* and mouse brain *in vivo* was determined. NPs of 114 nm in size diffused within the human and rat brain, only when they were densely coated with PEG. These findings were confirmed *in vivo* in mice, where 40- and 100-nm, but not 200-nm, NPs rapidly distributed within brain tissue, only if densely coated with PEG. Similar results were observed in rat brain tissue with paclitaxel-loaded biodegradable NPs of similar size (85 nm) and surface properties [137]. Doxorubicin-loaded NPs made of naïve albumin (HSA) plus cationic- (c-HSA) or mannose-modified-albumin (m-HSA) were developed with the goal of traversing the BBB and targeting brain tumors. The c/m-HAS-NPs with size 90.5±3.1 nm and zeta-potential of -12.0±0.3 mV at c- and m-HSA feed ratios of 5% and 10%, respectively, were tested. The c/m-HAS-NPs displayed good stability over 3 days based on particle size and a linear gradual doxorubicin release over 2days. Specifically, the inhibitory concentration (IC_{50}; 0.5±0.02μg/ml) of c/m-HSA NPs was >2.2-15.6 fold lower than those of doxorubicin or the other HSA NPs. Moreover, c/m-HSA NPs were localized to a greater extent in brain glioma compared to naïve HSA NPs. This improved anti-glioma efficacy of NPs was due to the dual cationic absorptive transcytosis and glucose-transport mediated by the combined use of c- and m-HSAs [138].

Overall, surface charge affects the fate of brain-targeted nanoformulations in biological systems in terms of their toxicity, circulation stability, biodistribution, and cellular uptake. Hence rational design and appropriate characterization methods of nanoformulations surface charge may help in developing more advanced and effective therapeutics to brain diseases.

4.5. Surface functionality

Surface chemistry/functionality plays an important role to facilitate the NPs BBB penetration. Such functionalities can be grouped into several categories [134].

a. ***Molecules such as surfactants*** that mediate the adsorption of blood proteins interacting with receptors and/or transporters in the brain endothelium and enable the NPs crossing of the BBB. For example, polysorbate 80 and poloxamer 188 surfactants have shown the ability to assist NPs to deliver drugs across the BBB following intravenous injection. These surfactants on the surface of NPs adsorbed Apo A-I and/or Apo E from the blood on to the NP surface, which induced receptor-mediated endocytosis followed by transcytosis to cross the BBB [139,140].
b. ***Molecules categorized as targeting ligands*** that directly interact with BBB receptors and/or transporters (Fig. 5) such as transferrin receptor, insulin receptor, glucose transporter, GLUT1 or albumin transporters, lactoferrin (Lf receptors), and low-density lipoprotein receptor–related protein (LRP) [141–143]. Conjugating surface ligands onto the surface of nanocarriers allow enhanced and targeted drug delivery, however, the number of ligands, as well as their receptor affinity, has an important impact in the transport of NPs across the BBB [144]. Conjugating a high number of ligands (high avidity) on NP surface can hinder endocytosis by endothelial cells, and make the NPs remain attached to the endothelial cell plasma membrane. Hence, NP avidity must be modulated for effective BBB transcytosis since high avidity will hinder NPs bound to the receptor to be released into the brain parenchyma. GNPs conjugated with high concentrations of transferrin (100–200 molecules per NP) stay bound to brain endothelial cells compared to gold NPs conjugated with low concentrations of transferrin (20-30 molecules per NP) which interacted effectively with the receptor, underwent transcytosis and released into brain parenchyma [144]. Pang *et al.* evaluated the effect of the number of mouse-anti-rat monoclonal

antibody ligands (OX26) on the distribution of polymer NPs (PEG-PCL-based polymersomes) and brain delivery properties [145]. Results showed an inverse correlation between the amount of OX26 ligand per NP (0, 5, 34 and 92) and the half-life in the blood. The optimized number of OX26 conjugated per polymersome to provide the greatest BBB permeability was 34. However, by further increasing the ligand number to 92, the distribution of NPs in the brain was decreased.

The charge and length of the targeting ligand can also impact the blood circulation of nanocarriers. For example, the immune-compatibility of brain-targeted liposomes was enhanced through the modification of charge and length of targeted ligand [146]. Three types of brain-targeted peptides of the same length, but with different net positive charges were synthesized. The level of IgM adsorption onto liposomes was greatly enhanced with the increase of net positive charges of peptides. To evaluate the impact of ligand length on IgM adsorption, peptides with 8 and 16 amino acid residues were synthesized, while both possessed the same net positive charges. The results demonstrated that 8 amino acid residues-conjugated liposomes showed a 4-fold decrease of IgM adsorption compared to 16 amino acid residues conjugated-liposomes. The present study brings insights into the development of ligand-targeted nanocarriers by correlating the positive charge and length of targeted peptide ligand in precisely modulating their protein adsorption and composition of protein corona formation in blood circulation.

c. ***Molecules that improve systemic circulation*** of NPs in a physiological environment. Blood proteins rapidly adsorbed to NP surface forming a "protein corona" (discussed later in this manuscript), which may accelerate systemic clearance of the NPs through the reticuloendothelial system (RES) and decrease the NP dose available for accumulation in the targeted sites. The most common approach to overcome this issue is to use molecules such as PEG, due to its near neutral and hydrophilic nature, and surfactants (e.g. poloxamers) that minimize the protein interaction to NPs. For example, polystyrene NPs (below 200 nm) coated with PEG (5 kDa; 9 PEG molecules *per* 100 nm^2) were able to cross the BBB due to its improved blood circulation time, and PLGA NPs (78 nm) coated with PEG could rapidly penetrate rat brain tissue *ex vivo*, in contrast with uncoated NPs [137].

4.6. Dissolution, solubility and drug release profile

The effectiveness of a delivery system is dependent not only on its therapeutic components but also on its solubility and diffusion. When the drug is delivered using nanocarriers, therapeutic effectiveness is affected by particle size, shape, and morphology, which directly or indirectly affect the drug release process (also affected by the biodegradation of the particle matrix (polymers). Thus, control of the particle size of NPs provides a means of regulating drug release rates. In general, the smaller the particle size, the larger the surface area-to-volume ratio; hence, most of the drug would be at, or near, the particle surface which leads to faster drug release. In contrast, larger size particles have large cores, which allow more drugs to be encapsulated and can give long-term release. There may be a burst release of drugs if they are weakly bound to NPs surface followed by a sustained release profile. The release of drugs from the nanoformulation also depends on pH, temperature, drug solubility, drug diffusion through the NP matrix, matrix/surface swelling and erosion, types of polymers used in NPs preparation, NPs interaction with the biological system, and the combination of these processes. Moreover, the ionic interactions between the drug and NPs polymer matrix may inhibit or slow down the drug release [147–149].

4.7. Hydrophobicity

Hydrophobicity of nanoformulations has a critical role in various biological processes such as protein adsorption, cellular uptake, and immune response. Cerium oxide (CeO_2), silica-coated CeO_2, barium sulfate ($BaSO_4$) and zinc oxide (ZnO) NPs exhibited different lung toxicity and pulmonary clearance, possibly because they acquired coronas with different protein compositions due to the differences in NP surface chemistry, charge, and hydrophobicity that in turn influenced NP-biomolecular interactions [150]. Generally, hydrophobic nanocarriers adsorb more proteins than the hydrophilic one, which may cause agglomeration and higher opsonization, leading to shorter systemic circulation time than hydrophilic NPs [151]. However, only a few methods are currently available for characterizing the hydrophobicity of nanocarriers [152,153]. Moreover, these methods are not applicable to all types of nanocarriers and involve expensive and time-consuming techniques. To overcome the limitations of current methods, NPs hydrophobicity was quantitatively characterized by measuring their affinity towards specifically functionalized surfaces [154]. The determination of the affinity of NPs towards substrate surfaces of different hydrophobicity enabled the direct characterization of the NPs having unknown surface functionalization and residual hydrophobicity.

4.8. Colloidal stability

Analyzing the colloidal stability of nanocarriers in biological environments is critical to define their fate after administration. When introduced into complex biological media, nanocarriers get exposed to biomacromolecules, electrolytes, proteins, lipids, etc., which may aggregate them, modify the intrinsic properties and *in vivo* behavior [155]. Nanoformulations aggregation may significantly alter their cell uptake, cytotoxicity, as well as pharmacokinetics and biodistribution. Transferrin-coated gold NP aggregates of different sizes were synthesized and their uptake and toxicity in three different cell lines were evaluated [156]. There was a 25% decrease in uptake of aggregated NPs with HeLa and A549 cells in comparison to single and monodispersed NPs. However, there was a 2-fold increase in MDA-MB 435 cell uptake for the largest aggregates. These results highlighted the need to investigate the behavior of aggregates with cells on a case-by-case basis. In this regard, for brain delivery of NPs, it is important to systematically characterize the biological behavior of brain-targeted NPs as a function of ion composition, concentration, and pH, especially in the systemic circulation and biological fluids such as cerebrospinal fluid (CSF). In one of the recent studies, PEG-coated and carboxyl-coated polystyrene (PS-PEG and PS-COOH, respectively) NPs were used to evaluate the aggregation kinetics, colloidal stability, and diffusive capability in conditions relevant to the brain microenvironment. Size, surface charge, and surface coating were varied in a range of CSF ion concentrations and compositions, pH conditions, and temperatures. Results confirmed that small changes in calcium concentration and pH destabilized the NPs in CSF. However, PS-PEG NPs remained stable over a wider variety of conditions than PS-COOH NPs, and had higher diffusion capabilities in both agarose gels, an *in vitro* model of the brain microenvironment, and an organotypic brain tissue slice model [157]. These kinds of studies are important and should be performed on a regular basis for each type of nanocarrier system, which may demonstrate the need for steric stabilization to maintain nanoformulation colloidal stability in a range of conditions.

While in the systemic circulation, the nanocarriers are primarily cleared by the RES system consisting of phagocytic cells, such as monocytes and macrophages, which can engulf and remove the nanocarriers through the process of opsonization [158]. The destabilization and removal of the colloidal NPs is significantly related to the protein–NP interaction through the process of opsonization [158]. Steric stabilization is the most commonly used approach to increase the stability of NPs in the systemic circulation. It has been demonstrated that

protein adsorption is minimized, or not observed when the NPs are coated with hydrophilic and neutral surfactants and polymers (e.g. PEG, poloxamers) [158]. For example, gold NPs and PEGylated gold NPs with 20-30-nm or 50-nm size were studied to avoid the influence of particle diameter, and their biodistribution evaluated in the mice. Gold concentrations in brain, heart, lungs, liver, stomach, pancreas, spleen, kidneys, blood, urine, and feces were measured up to 48 h after administration. At 48 h after intravenous administration, accumulation in the liver and spleen was significantly reduced by PEGylation, and the gold amounts of PEGylated NPs with 20-30 nm and 50 nm sizes in the brain were 3.6 times and 2.7 times higher than those of bare gold NPs, respectively [159].

Overall, several parameters such as size, shape, surface charge, hydrophobicity, surface chemistry, etc., influence the transport of nanoformulations through the BBB at different extents. The interaction of the NPs with endocytosis mechanisms (clathrin-mediated and caveolin-mediated) plays a relevant role in their ability to successfully cross the BBB. In general, NPs with size ≤ 200 nm have more chances to efficiently cross the BBB since this is the limiting size for an NP to undergo endocytosis through a clathrin-mediated mechanism [160]. The shape of NPs can vary from spherical, cubic, rod-like, among others, however, most of the studies have been performed with spherical NPs since they are relatively easy to prepare. Positively charged NPs can use the adsorptive transcytosis pathway more easily than their neutral or negatively charged counterparts due to the negative charge of the endothelial cell membrane. On the other hand, neutral or negatively charged NPs showed reduced protein adsorption, leading to longer circulation times which may also be achieved by coating the NPs with compounds such as PEG. However, neutral and negatively charged NPs (low concentrations) did not affect the integrity of BBB, whereas the BBB was disrupted in the presence of positively charged NPs and high concentrations of negatively charged NPs. Although not much has been done in evaluating the role of nanoformulations elasticity in brain-targeted drug delivery approaches, a few studies clearly suggested that elasticity is an important parameter in determining the NPs ability to cross the BBB. NP surface decoration with different ligands increases BBB crossing of the NPs by taking advantage of transport- and receptor-mediated transcytosis. However, ligands avidity plays an important role and needs to be optimized.

In summary, the application of nanomedicines for drug delivery across the BBB has demonstrated tremendous potential. However, based on above examples and since there are several factors affecting the biological behavior of nanoformulations, we still need to increase our knowledge of the precise interactions among the different factors related to nanoformulations physicochemical properties, leading to successful BBB transport and brain delivery. A better understanding of these factors will contribute to a more successful design of NPs that would be able to efficiently deliver therapeutic and/or diagnostic molecules to the CNS.

5. Nanocarrier properties and behavior in biological environment

As a new phase in the development of effective nanocarriers, biomedical engineers need to understand better how these systems behave in a biological environment, and how such an environment affects their interaction with cells [161]. In other words, it is important to model as objectively as possible, the downstream behavior and physiological processing of nanoformulation in a body with specific pathologies. Nanocarriers can substantially differ in their biophysical and biochemical properties (including size, ionic charge, surface hydrophobicity, apparent shape surface area, etc.) depending upon the biological fluid where they are administered and “traveling”. Better characterization and understanding of how these properties influence nanoformulation in a biological milieu, from the stage of cellular experiments, should allow designing improved chemical and biological strategies to increase residence time, ability to cross biological barriers, and capacity to target the diseased tissues and to be uptaken by affected, not healthy, cells and tissues. These design strategies also need to take into account (a) the intended route of administration of the nanocarriers (e.g. intravenous, intramuscular, intraperitoneal, oral, intracranial, intracerebroventricular, intrathecal, intranasal, or intraocular delivery), and, consequently, (b) the biochemical characteristics of the physiological media and possible flow where these nanocarriers will be present and interact with, as well (c) the cells present in the environment such as the endothelial cells of the blood vessels [162].

During the development phase, experiments should be designed to understand the impact of the biological environment of cell cultures themselves, as those are typically used to assess the functional impact and cytotoxicity of nanocarriers on cells as well as their potential cellular uptake. In addition, cell cultures are now used as a production platform of naïve and drug-loaded EVs for therapeutic applications. Thus, understanding the influence of the cell medium and biological fluids components on these nanoformulations, synthetic or cell-derived, is therefore increasingly important. Some of these matrices are briefly discussed in the following sections/sub-sections.

6. Complex cell matrices and biological fluids

Increasingly, biomedical engineers need to design nanoformulation having in mind the specificities of the biological milieu(s) in which they are administered prior to reaching the targeted tissues or cells. This should be even regarded as a dynamic development process as nanoformulation may pass through different biological compartments to reach their intended target site. For instance, intravenous nanoformulation targeting the brain should circulate in the blood and pass through the BBB prior to reaching the CSF and the affected brain compartment. It has been recently shown that NPs may dramatically change their surface characteristics when crossing the BBB [163]. The complexity in nanoformulation design can be further complicated as biological fluid compositions may exhibit differential properties among healthy individuals and patients. Understanding how the protein composition of biological fluids and any variations associated with pathological states may affect nanoformulation properties is, therefore, a needed development field in nanomedicine. The complexity of the task is further complicated by the fact that biological fluids, as well as cell cultures, contain a plethora of EVs which may themselves potentially interfere with administered nanoformulation.

6.1. In vitro matrices

Nanoformulations are evaluated *in vitro* on cell cultures as a means to assess their toxicity as well as interactions and internalization with cells [164,165]. The cell culture basal media typically used are usually supplemented by 5-20% of nutritive protein material, such as fetal bovine serum (FBS), that may significantly influence nanoformulation properties and biological behavior [155,166].

6.1.1. Culture media

Depending on the cell type, media composition can vary based on the metabolic and nutritional needs of different cells. This may impact the colloidal stability of NPs *in vitro*. Table 1 shows the composition of some of the most common culture media such as Dulbecco Modified Eagle's Medium, DMEM; Minimal Essential Medium, MEM; or Roswell Park Memorial Institute Medium, RPMI) and human plasma. These media are very different in their ion composition, pH, amino acids, and the presence of proteins and lipids (reproduced with permission from [155]). This further complicates the interaction of NPs with their *in vitro* environment. Moreover, depending on the serum source, levels of lipids differ. Considering the variable composition of culture media, characterizing NPs in water or buffer/isotonic solutions does not represent the colloidal system used under *in vitro* conditions.

Table 1
Major components of commonly used cell culture media and human plasma (reproduced with permission from [155]).

Classification	Component	Unit	DMEM + 10% FBS	MEM + 10% FBS	RPMI-1640 + 10% FBS	Human plasma
Amino acids	Total	(mM)	10.65	5.43	6.44	2.32–4.05
Vitamins	Total	(mM)	0.15	0.04	0.24	<0.07
Cations	Sodium, Na^+	(mM)	155.31	144.44	124.27	142.00
	Potassium, K^+	(mM)	5.33	5.33	5.33	4.00
	Calcium, Ca^{2+}	(mM)	1.80	1.80	0.42	2.50
	Magnesium, Mg^{2+}	(mM)	0.81	0.81	0.41	1.50
	Iron, Fe^{3+}	(mM)	0.25	n/a	n/a	10.00–27.00
Anions	Chloride, Cl^-	(mM)	117.47	124.37	100.16	103.00
	Bicarbonate, HCO_3^-	(mM)	44.05	26.19	23.81	27.00
	Sulfate, SO_4^-	(mM)	0.81	0.81	0.41	0.50
	Nitrate, NO_3^-	(mM)	0.74	n/a	0.85	20.00
	Phosphate, PO_4^{3-}	(mM)	0.92	1.01	5.63	1.00
Proteins	Total	($g\ L^{-1}$)	3.00–4.50	3.00–4.50	3.00–4.50	65.00–80.00
	Serum albumin	(mM)	0.05	0.05	0.05	0.58
	α-Globulins	($g\ L^{-1}$)	0.30	0.30	0.30	8.10
	β-Globulins	($g\ L^{-1}$)	0.27	0.27	0.27	11.50
	γ-Globulins	($g\ L^{-1}$)	0.07	0.07	0.07	15.60
	IgG	(mM)	$3.56 \times 10^{-0.05}$	$3.56 \times 10^{-0.05}$	$3.56 \times 10^{-0.05}$	0.08
Parameters	pH range		7.00–7.40	7.00–7.40	7.00–7.40	7.34–7.42
	Osmolality	($mOsm\ kg^{-1}$)	320–360	280–320	270–310	276–29

Several factors determining the formation and compositions of NPs protein corona have been identified, but the effect of the composition of media on the composition of protein corona has not been much evaluated. A recent study has determined the effect of dispersion media on the composition of protein corona of polyacrylic acid-coated cobalt ferrite NPs and silica NPs [167]. Results confirmed the effect of the dispersion media on the protein corona composition and the differences between constituents of the media used for dispersion of NPs, such as divalent ions and macromolecules, were responsible for the differences in protein corona composition formed in the presence of FBS. Hence, the composition of dispersion media is an important factor to consider in evaluating protein corona on nanoformulations.

6.1.2. Fetal bovine serum (FBS)

The gold standard supplement for *in vitro* cellular assays, FBS, is obtained by letting fetal blood, collected without anticoagulant, to clot. Albumin is quantitatively the main protein found in FBS (ca 30 mg/mL), but many other proteins are present, for a total protein content close to 40-45 mg/mL, while the quantity of IgG, contrarily to adult human serum, is low in FBS [168]. Heat inactivation of FBS is performed for complement inactivation/depletion, and contributes to precipitation and removal of heat-sensitive serum proteins, impacting NPs protein binding and cellular uptake compared to medium supplemented with non-heat-inactivated serum [169]. Standard FBS contains EVs and it seems preferable to select EV-free FBS or to perform a removal step of EVs by ultracentrifugation [170] or ultrafiltration [171] to avoid any possible risks of interference when assessing nanoformulations. Table 2 summarizes the mean composition of FBS and other biological fluids, including proteins potentially relevant to protein corona of nanoformulation for brain administration. Substantial variations in the composition may exist among batches and suppliers [172].

6.1.3. Human plasma

Plasma is, like FBS or HPL, a complex biological matrix obtained by centrifugation of anticoagulated blood. Its mean total protein content ranges between 60 and 70 mg/mL (Table 2). The diversity in plasma proteins, which account over 1000 entities, encompasses the level of abundance (from ng/mL to ca.40 g/mL), molecular mass (from a few thousand to several million Daltons), isoelectric point (from very acidic to basis), or functional activity [173]. Albumin accounts for about 60% of all plasma proteins in terms of mass, for a content close to 35 to 40 mg/mL. The second most abundant class of plasma proteins is immunoglobulin (Ig). IgG is the most prominent of all, at a mean dose of approximately 12.5 mg/mL, that can increase to 15 to 18 mg/mL when donors (or patients) are exposed to an immunologically challenging environment. IgA is present at a mean level of ca. 1 mg/mL and IgM ca. 0.5 mg/mL. Fibrinogen, which is the protein responsible for the formation of a fibrin clot and control of bleeding, circulates at ca. 2 to 3 mg/mL. The plasma proteome is made of a myriad of other proteins present in less abundant quantities [173]. These include several protease inhibitors (antithrombin, C1 esterase inhibitor, alpha 1-antitrypsin, inter-alpha-trypsin inhibitor, alpha 2-macroglobulin) circulating in quantities varying from ca. 100 mg/mL to 1.5 g/mL. The anticoagulant proteins (protein C and protein S), and various coagulation factors, in addition to fibrinogen (Factors II, V, VII, VIII, IX, X, XI, XII, XII) are found at a few ng or hundreds of ng per mL. Most coagulation factors are present in plasma in an inactive form, known as zymogens, and act as precursors of proteolytic enzymes that contribute to the coagulation cascade. They are very reactive once activated by various triggers both *in vivo* (such as the exposure of tissue factor) or *ex vivo,* by the contact with activation surfaces, such as glass, and potentially some nanoformulation. Small molecular weight proteins and peptides present in plasma include various cytokines and growth factors, such as insulin -like growth factor (IGF), present at ng/mL levels.

While most plasma proteins have a molecular mass in the 30-180 kDa range (which would correspond to an apparent size of 5~12 nm, some are actually much bigger, like fibrinogen (330 kDa), alpha 2 macroglobulin (725 kDa), IgM (950 kDa), and most particularly Von Willebrand factor which can form ultra-high molecular mass multimeric complexes of several million Da. At the physiological range of blood of pH 7.4 ±0.05, a higher proportion of plasma proteins are negatively charged as their isoelectric point (Pi) is in the pH range of pH 3.8 to pH 6.8. For instance, albumin Pi is 4.7. Other proteins have a Pi between pH 7.7 and pH 10 and are therefore positively charged at the pH where cell cultures are conducted. For instance, Ig(s) and factor XI, a pro-thrombogenic coagulation factor, have a Pi close to pH 8. A very small proportion of proteins have a Pi near pH 7.4 as this would make them prone to precipitation or aggregation [174]. Some plasma proteins, such as albumin, are hydrophobic and are capable to bind fatty acids and hormones, and could adsorb on hydrophobic nanocarrier [175]. It should not be underestimated that plasma prepared by standard centrifugation of whole blood is not cell -free. Residual platelets can be present at 10-40 x 10^6/mL and white blood cells at ca. 10^6/mL [176]. In addition, functional EVs are present in plasma at a dose typically over 10^8/mL [177,178]. When whole plasma is used as a medium supplement, heparin anticoagulant may be needed to avoid medium gelation during cell cultures. The negative charge of heparin and affinity for some coagulation factors and growth factors may interfere with

Table 2
Physiological characteristics and mean composition of biological fluids, including proteins, potentially relevant to protein corona of nanoformulations for brain administration.

Parameters	Biological fluids						
	Fetal bovine serum [301]	Anticoagulated plasma [302]	Human serum [197]	Anticoagulated human platelet lysate [181,303]	Serum converted Human platelet lysate [181,197]	Cerebro-spinal fluid [192]	Tears [197,304]
pH	7.2-7.4	7.0-7.4[a]	7.2-7.4	7.0-7.4[a]	7.0-7.4[a]	7.2-7.4	7.2-7.4
Osmolarity, mosm/L	290-310	290-310	290-310	290-310	290-310	270-290 [305]	290-310
Anticoagulant	_	See Table 3	-	See Table 3	See Table 3	-	-
Mean total proteins mg/mL	30-45	55-65	60-70	50-65	58-70	0.15-0.50	7-8
Abundant proteins (mean concentration; mg/mL)	Albumin (25-35) IgG[b]	Albumin (40) IgG (12.5) Fibrinogen (3)	Albumin (40-45) IgG (12.5)	Albumin (40) IgG (12.5) Fibrinogen (3)	Albumin (40-45) IgG (12.5)		Lysozyme (2-2.5) Lactoferrin (1.5) IgA (0.41)
Other relevant proteins (mean concentration; mg/mL)	Protease inhibitors, Complement system[c], hemoglobin (0.11);	Protease inhibitors: alpha 2 macroglobulin (2.6); Alpha 1-antitrypsin (1.5); C1-esterase inhibitor (0.17); antithrombin (0.1); alpha 2 antiplasmin (0.07) Fibrinolytic proteins: plasminogen (0.2); histidin rich glycoprotein (0.1) Complement system Apolipoproteins		Protease inhibitors: alpha 2 macroglobulin (2.6); Alpha 1-antitrypsin (1.5); C1-esterase inhibitor (0.17); antithrombin (0.1); alpha 2 antiplasmin (0.07) Fibrinolytic proteins: plasminogen (0.2); histidin rich glycoprotein (0.1) Complement system Apolipoproteins		Protein transferred from the brain and blood as a function of molecular mass; relative enrichment in transthyretin and Cystatin C	Albumin (0.05) IgG (0.032) Fibronectin (0.02)
Coagulation factors (μg/mL)	Consumption due to clotting	Coagulation factors II (150); V (7); VII (0.5); VIII (0.3); IX (5); X (10); XI (5); XII (40); Von Willebrand Factor (10)	Consumption due to clotting	Coagulation factors II (150); V (7); VII (0.5); VIII (0.3); IX (5); X (10); XI (5); XII (40); Von Willebrand Factor (10)	Consumption due to clotting	Presence of some lower mass pro-coagulant factors [190]	-
Hormones, growth factors (ng/mL)	Growth hormone (39); cortisol (0.5); Parathormone (1.7); Triiodothyronine, T3 (ng/ml) 1.2; Thyroid-stimulating hormone (1.22); Follicle-stimulating hormone (0.09)	IGF-1 (10-50)		PDGF-AA, -BB, -AB; IGF-1; TGF-ß; BDNF; VEGF; HGF (~1-100) [180,181]	PDGF-AA, -BB, -AB; IGF-1;TGF-ß; BDNF; VEGF; HGF (1-100) [180,181]	IGF-1, NGF, PDGF, VEGF (doses vary with brain pathologies)	EGF (1.6) TGF-alpha (0.2) TGF-ß1 (2)
Blood and other cells	Absent	Residual red blood cells, white blood cells, and platelets[d]	_	Residual red blood cells, white blood cells, and platelets[d]	_	Very few cells (0-4/μL)	_
Extra-cellular vesices	Present [306,307]	Present [211]	Present [307]	Present [308]	Likely present [181]	Present [309]	Present [310]

[a] Slight acidification due to citrate anticoagulant solution.
[b] IgG content is typically low; FBS brands poor in IgG are available.
[c] Removed by heat-inactivation at 56°C,30 min.
[d] Variable upon mode of preparation.

the analysis of the biological impact of nanoformulation and cell growth. In addition, heparin was found to influence the uptake of polystyrene NPs into macrophages and prevent internalization into HeLa cells [179].

6.1.4. Human serum

Human serum is obtained, as is FBS, by letting blood, collected without anticoagulant, to clot due to the generation of thrombin that converts fibrinogen into fibrin and degranulates the platelets. Clotting results in depletion in fibrinogen and coagulation factors and enrichment in platelet-derived growth factors (that can be found at a content of a few ng/mL) [180]. The content in albumin, Ig remains essentially unchanged compared to that of plasma, and serum total protein content is about 10% less than plasma (Table 2). The clotting process contributes to the entrapment of blood cells but also to the release of EVs in particular from platelets [178]. The content in IgG (ca. 12.5 mg/mL) of human serum prepared from adult blood is much higher than that of bovine serum made from calf blood.

6.1.5. Human platelet lysates (HPL)

Translational evaluations of nanoformulation are increasingly using cell culture assessment methods where HPL is preferred as growth medium supplement for cell cultures as it mimics better the physiological conditions found under clinical use, and is more potent that FBS for cell growth stimulation. Also, using HPL as growth medium supplement for cells used for the generation of EVs for therapeutic applications presents immunological and pathogen safety advantages compared to FBS [181]. HPL is produced from licensed platelet concentrates for transfusion, and has protein content close to 50-65 mg/mL with albumin and Ig(s) being the most abundant proteins (Table 2). The protein composition, in particular the content in fibrinogen and coagulation factors, depends on the HPL production process, in particular whether or not a serum-conversion step, which removes fibrinogen, is performed [181–183] (Table 2). When HPL is not serum-converted, an addition of heparin anticoagulant (2-3 IU/mL, final concentration) may be required in order to avoid culture medium gelation and artefactual cell death [181,182].

6.2. In vivo biological fluids

It is important to have a general vision of the impact of various biological fluids on nanoformulations as a means to develop optimal DDS for a given targeted application. Therefore, this section summarizes the characteristics of various body fluids and how those can affect nanocarriers' properties.

6.2.1. Whole blood and blood cells

Understanding how the blood components can affect *in vivo* properties and dynamics of nanoformulations intended for brain delivery and most particularly their capacity to cross the BBB is key to the improvement of nanocarriers. The volume of blood in an adult human is close to 4~5 liters highlighting the fact that upon intravenous administration nanocarriers are dispersed into a large volume, decreasing their concentration at the interface between blood and the BBB. In addition, nanoformulations are exposed to the risk of (a) clearance through phagocytosis by the RES, (b) surface modifications in contact with the blood proteins, and (c) cross-talks with reactive blood cells, which can all potentially affect, or influence, the capacity to cross the BBB. Understanding the interface existing between the blood and the brain compartments is crucial as the human brain is irrigated by ±644 km of blood vessels, most of them capillaries that account for about 12 m^2 cell surface area. The mean inter-capillary distance is approximately 40 μm, facilitating the quick passage and equilibrium of small molecules and solutes between the blood and the brain interstitial fluid [184,185].

The mechanisms in the exchanges through the BBB involves (a) pinocytosis and bulk flow fluid transcytosis for solutes, (b) transmembrane diffusion for small molecules and lipid-soluble compounds less than 400 kDa, (c) carrier-mediated transport for components like carbohydrates, nucleotides or hormones, (d) receptor-mediated transcytosis for trans-endothelial bi-directional transport of proteins [163]. However, some of these pathways can be partially altered in brain disease states [163], making even more complex any predictive assumptions of the capacity of a drug or its nanoformulation to reach the pathological site in the brain.

Blood is truly a very complex biological material, under constant flow and exposed to shear stress, that comprises various cells with dedicated physiological functions, circulating in the plasma compartment that contains multiple peptide, cytokines, proteins, amino acids, hormones, lipids, and electrolytes. Blood composition and reactivity to external factors, such as infused NPs, is important to understand since nanoformulation are often transfused intravenously, and are therefore immediately in contact with the blood components that have the capacity to immediately interact with nanocarriers and modify their surface properties, especially under flow. Therefore, once nanocarriers are injected, blood directly impacts the dilution factor, life span, elimination rate, targeting, or capacity to cross biological barriers of nanocarriers. In addition, the blood composition should be regarded as a dynamic process, evolving in a continuous manner due to an active permanent synthesis of new blood cells and proteins. This dynamic process can therefore permanently expose nanoformulation to naïve cells and plasma proteins (see section above) further influencing pharmacokinetics and pharmacodynamics of nanocarriers. The level of complexity is further enhanced by the possibility that the nanocarrier may contribute, through the constitution of the protein corona, to the delivery of biologically active, potentially toxic, blood components to the brain [163].

Blood cells are produced in the bone marrow through a continuous physiological generation process called hematopoiesis. The cellular compartment represents about 55% of the blood volume. The most abundant cells, by far, are the RBCs (erythrocytes), which are anucleated, and present at a mean content of approximately 5 x 10^6/μL, with a mean size of 10 μm. They contain mainly hemoglobin and some enzymes. Their life span is about 120 days, after which they are removed from the blood circulation by the spleen, liver or bone-marrow. Platelets (or thrombocytes) are also anucleated cells. They circulate in blood at approximately 0.3~0.4 x 10^6/μL, with a mean size of 2~3 μm. Platelets are complex cellular entities with an alpha-granules proteome rich in growth factors and neurotrophins, coagulation factors, and adhesion molecules, and dense granules containing neurotransmitters such as dopamine, serotonin, or epinephrine as well as ADP and ATP [181,186]. After a life-span of 7~10 days, apoptotic platelets and platelet debris are up-taken by macrophages present primarily in the spleen and liver, and possibly also by those present in the lung [187]. The platelet membrane is decorated by a complex set of bioreactive glycoproteins that serve to anchor (activated) coagulation factors (such as thrombin, factor VIII, fibrinogen, von Willebrand factor) and contribute to their primary function leading to the formation of the hemostatic fibrin clot in case of hemorrhage. Platelets are equipped with a canalicular system that connects alpha granules with the fluidic environment and allows the capture of biomolecules and possibly as well NPs of a size of 20 nm or more. The white blood cells (WBC; leucocytes) are nucleated cells present in various number, ca. from 0.004~0.011 x 10^6 cells/μL of blood, influenced by health or physiological situations, with a size of 8~30μm depending upon their type. The leucocytes are divided into two main categories. The granulocytes comprise neutrophils, basophils and eosinophils, while the agranulocytes encompass monocytes and lymphocytes, themselves divided into T-cells, B-cells, and NK-cells.

6.2.2. Cerebrospinal fluid (CSF)

The volume of the CSF in an adult human is about 125 to 150 mL [188]. It is a complex biological milieu that, however, substantially differs from blood and plasma by being essentially acellular, apart from the presence of some white blood cells and with a much lower total protein content (ranging from 0.15 to 0.45 mg/mL) in healthy individuals (Table 2). Proteomics analyses have, unsurprisingly, revealed an analogy of composition between CSF and plasma [189], including the presence of lower molecular mass pro-coagulant factors [190], but also that of proteins derived from the brain, such as acetylcholinesterase, and spinal cord [191]. The CSF proteome contains over 300 polypeptides as detected by 2D-electrophoresis [191] and its composition may evolve in patients with neurodegenerative disorders due to the presence of proteins released from the brain [189]. There is a regular constant renewal of the CSF [188] which implies that, here also, the behavior of nanoformulation should be regarded with a dynamic perspective. Nanoformulation development should also consider the fact that the CSF of Alzheimer disease patients has altered and higher protein composition and content [192], most particularly among the group of apolipoproteins (Apo) [193].

6.2.3. Tears

Nanoformulation may be administered as eye drops to treat alterations of the corneal endothelium or dry eye syndrome, and to deliver nanoformulation to the retina in order to avoid an administration into the vitreous humor. However, ocular administration has also been shown to be a possible route of administration of drugs to the brain [194,195]. Still, topical eye administration of a (non-encapsulated) recombinant traceable form of nerve growth factor in a mice model of Alzheimer disease was found significantly less potent, even at higher doses, than when using intranasal administration [196]. The composition of tears shares several similarities with that of plasma, including bio-physicochemical parameters like osmolality (ca. 300 mOsm/L), pH (7.2-7.4), presence of electrolytes, and proteins [197]. Sodium ions (145 mEq/L) are at similar levels. There is however about five times more potassium ions (24.1 mEq/L), but less calcium (1.5 mEq/L) and phosphate (24.1 mEq/L) ions. One main difference of relevance to the impact on nanoformulation is the total protein content that is approximately 10 times less (ca. 7-8 mg/mL) in the ocular fluid than in plasma, thereby decreasing, in principle, any potential impacts on the properties of nanocarriers and protein corona formation. Proteins in the ocular fluid (Table 2) include anti-microbial lysozyme (2-2.5 mg/mL), anti-inflammatory lactoferrin (1.5 mg/mL), albumin (ca. 0.05 mg/mL),

fibronectin adhesion protein (21 μg/mL) as well as components of the complement system. IgA (ca. 0.4 mg/mL) constitutes the main Ig class and contributes to protection against infections. Vitamin A (16-20 ng/mL) is less than in plasma but vitamin C (ca 110 μg/mL) and glutathione (ca. 100 μM), which exert an antioxidant role, are in higher content. Several growth factors such as TGF-β1, PDGF, EGF, HGF, and VEGF are present in the ocular fluid [198].

6.2.4. Aqueous humor

The aqueous humor is present in the anterior and posterior chambers of the eye and provides nutritional elements to the avascular lens. Administration of nanoformulation into the aqueous humor is performed to repair damages of the corneal endothelium. The aqueous humor exhibits an electrophoretic profile similar to that of plasma but its total protein content is less than 0.25 mg/mL [199–201], revealing the efficient effect of the blood- aqueous barrier (BAB) to isolate from the blood compartments [200]. IgG is present at 0.05-0.15 mg/mL, including in patients with cataract, and IgA and IgM at close to 0.04 mg/mL [202]. Proteomics studies have identified possible variations in the amount of albumin, cytokeratin type II and alpha 1-antitrypsin in patients undergoing acute corneal rejection [203]. Protein oxidation was found to be a physiopathological mark of pseudoexfoliation syndrome [204], and various protein changes as identified in 2D-electrophoresis were also found associated with retinoblastoma [205].

6.2.5. Vitreous humor

The vitreous humor is a unique biological gel structure, highly transparent and hydrated, found in the posterior ocular segment between the lens and the retina [206]. Nanomedicine products are administered in the vitreous humor to treat pathologies of the retina. The protein composition of the vitreous humor is characterized by the high content in collagen type II. There is a beneficial role played by glycosaminoglycans, in particular hyaluronic acid, chondroitin sulfate and heparan, in stabilizing its gel state [207]. Proteomics studies have identified that the protein composition of the vitreous humor is influenced by pathological conditions that include diabetic retinopathy and proliferative vitreoretinopathy, diseases that impact signaling pathways [206].

6.2.6. Synovial fluid (SF)

The SF is a viscous liquid that lubricates the cavities of synovial joints. Synovial administration is needed to treat diseases of the knee joints, in particular osteoarthritis. The SF contains a total of close to 20 mg/mL proteins deriving from plasma as well as cells making up the synovial membrane [208,209]. Proteomics studies have evidenced that SF contains numerous proteins including aggrecan, dermcidin, cystatin A, and inhibitor of cysteine protease, as well as numerous serine protease inhibitors which have been used in an attempt to discriminate between healthy SF and SF from OA patients [210]. Other proteins detectable in SF include albumin, various Apoproteins, thrombin, complement components, fibrinogen, fibronectin, transferrin, and vitronectin, among many others [210]. Some recent studies performed in dogs suggested that the total protein content of SF can increase substantially (up to 30-40 mg/mL) after inflammatory arthropathy [209].

6.3. Extra-cellular vesicles (EVs) in biological fluids

EVs are physiological subcellular structures that are present in all body fluids (blood, CSF, plasma, urine, tears, etc.) typically in very high amount. For instance, human blood plasma may contain well over 10^8 EVs/mL. Their content is under-evaluated in some techniques like flow-cytometry. Circulating EVs number increases in association with some pathologies like cancer and inflammatory diseases [177,211]. EVs are typically classified as exosomes, microvesicles and apoptotic bodies, depending upon the mechanisms that lead to their regeneration from cells [212,213]. Size of EVs ranges from ca. 30nm~150nm (exosomes), ca. 50nm~1μm (microvesicles) and ca. 100nm~5μm (apoptotic bodies), overlapping that of many nanoformulations (NPs and microparticles) [214]. The major common features of most EVs are to be delimited by a double layer phospholipid membrane, to express parental cell membrane markers, and to contain intracellular information (protein, lipids, miRNA, growth factors, and cytokines) that is representative of the cells from which they originate. There is evidence that EVs are not inert cub-cellular structures but are actually very instrumental in cell-cell communication and exert influential pathophysiological functions [215–219]. This cargo capacity is exploited in approaches where cell-derived EVs themselves are evaluated as DDS [214,220]. For instance, EVs from human universal group O RBCs are considered for large-scale drug delivery system of RNA drugs [221]. Therefore it appears increasingly relevant to consider how EVs can interact *in vitro* or *in vivo* with nanoformulations. So far, this aspect has, to our best knowledge, not been much taken into consideration in the understanding of the fate and mechanism of action of nanoformulations in biological fluids.

7. Protein corona formation: factors affecting and opportunities in brain-targeted nanoformulation design

7.1. What is the protein corona?

It is now well recognized that nanoformulations intended for translational medicine applications should be developed and studied taking into consideration the potential impact of the biological milieu in which they are studied and intended to be administered [222–224], One prominent event occurring is surface coverage of most, maybe not all NP [225], by a complex multilayer of proteins, called the "protein corona". A sudden switch of nanoformulation physicochemical characteristics may take place within seconds or minutes of contact with the biological matrix, altering their surface properties, potentially generating an immune response, and, hence, modifying fate, toxicity, and targeting [226–230]. Impact of protein deposition on NPs in cancer treatment field has been studied extensively and includes (a) masking of NP tumor targeting ligands and capacity to bind to tumor receptors, (b) enhancement of macrophage uptake reducing bioavailability, (c) increase in NP hydrodynamic size and (d) induction of aggregation preventing interaction with solid tumor microenvironment [231]. Understandably, the quantity, structure, and composition of the protein corona load is influenced by different factors (Fig. 6), including the biological environment where the nanoformulations is suspended, on one side, and the nature, size, curvature, porosity and surface chemistry of the nanoformulations, on the other [225,232]. This implies that the protein corona of a given nanoformulation will differ depending upon its immediate protein environment, and, in the same biological milieu, the protein corona will differ among nanoformulations.

It is apparent that NP protein corona plays a role in crossing several biological barriers, uptake, targeted delivery, etc., NPs crossing of the BBB should also be governed by the protein corona on their surface. For brain-targeted drug delivery approaches, an earlier study of using NPs with attached Apo E has enabled the delivery of drugs across the BBB. Results showed that only the NPs with Apo E were detected in brain capillary endothelial cells and neurons [233]. Nanoformulations based on colloidal systems (e.g. micelles or liposomes) can conjugate with plasma proteins and be eliminated quickly by macrophages of the liver and the spleen, thereby being unable to reach the BBB [4]. Another example is the change in physicochemical properties of superparamagnetic iron oxide NPs (SPIONs), depending on size and surface charge, when exposed to serum proteins, a situation leading to increased risks of toxicity-associated penetration of the brain through the BBB [234]. However, much research work is needed to mimic and model the evolution of the composition of nanoformulations from the stage of synthesis, administration to the blood, to their eventual delivery to the brain through the BBB, as most of these details are still unknown. The schematic of NP protein corona formation is shown in Fig. 6. However,

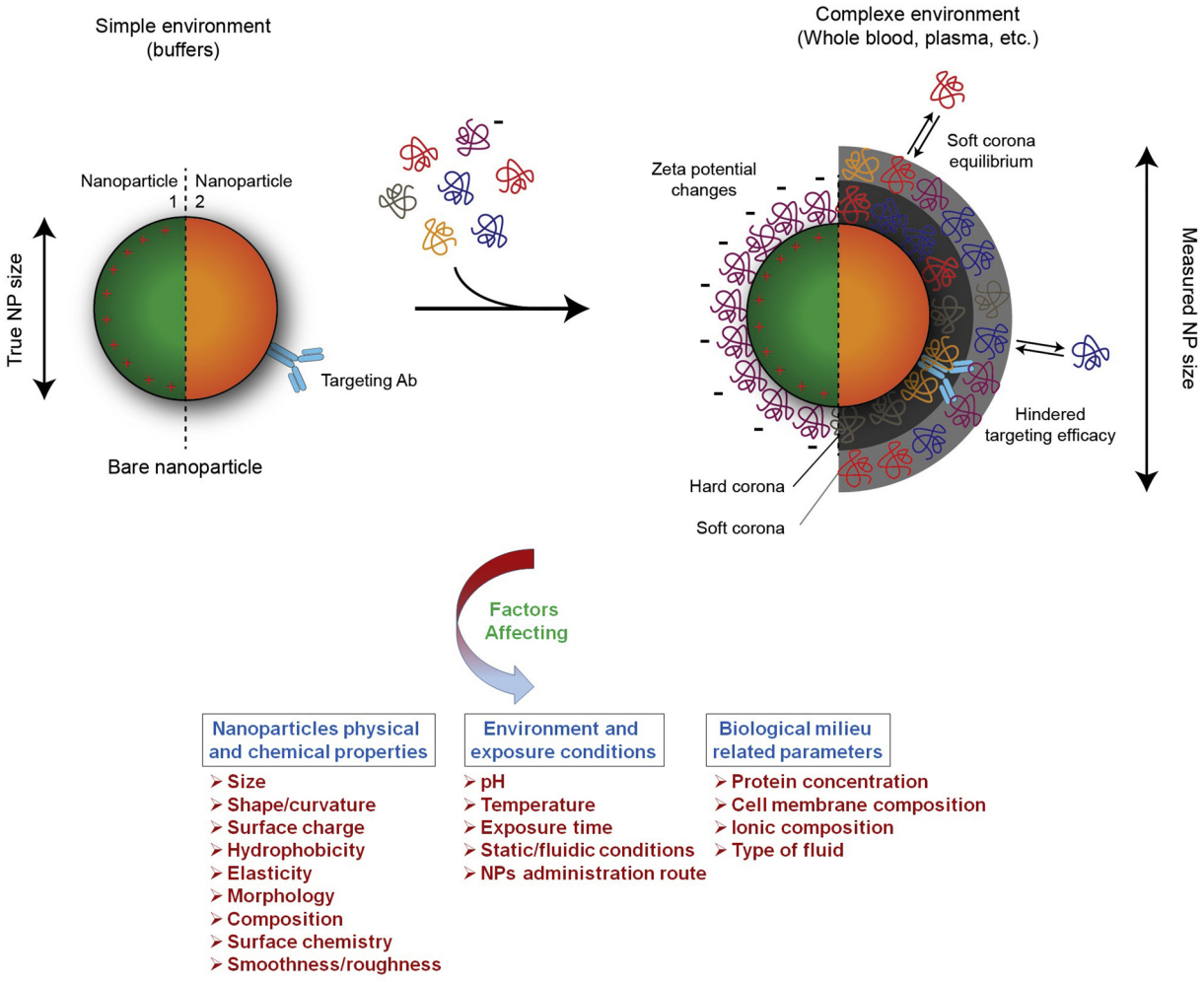

Fig. 6. Nanoparticle protein corona formation (soft and hard corona) and factors affecting its composition (nanoparticles properties-related, exposure condition-based, environment-based, and biological milieu-based factors).

much information is still lacking in the full understanding of the impact of the protein corona on nanoformulations behavior [235], as well as on the potential interfering effects of lipids in biological sources [236].

7.2. Dynamic structure of the protein corona

The protein corona is not a homogeneous entity but is formed by two compartments, one inner, which is highly adherent to the surface of the NPs ("hard corona"), and another ("soft corona"), on the outer core (Fig. 6), with a composition that fluctuates reversibly depending upon the composition of the immediate biological environment [151]. Thus, the protein corona, rather than being static in composition and organization, is an evolving dynamic biological entity reacting at least externally to its immediate microenvironment [237,238] and to flow (such as bloodstream), as occurs subsequent to intravenous administration [239], with impacts on cellular uptakes [240]. The crossing of tissue-to-tissue barriers, needed for NPs to reach a physiological target even affects the protein corona composition, as demonstrated using gold NPs and *in vitro* BBB model [163]. Thus, it is necessary to study the effect of protein corona on cellular level events, including NP-protein complex binding, internalization, and transportation, enabling rational design of brain-targeted delivery systems.

Complex, potentially personalized, analytic tools are options to consider for objective predictive assessment of functional benefits, toxicity, downstream behavior and physiological processing of nanoformulations in specific pathologies. This suggests increasing needs in using prediction modeling and computer simulation [151,175], machine learning tools [241] and artificial intelligence [242] in the development of "smart" nanoformulations designed for specific biological fluids and barrier crossing. As the interactions of the NPs with their immediate environment are necessarily guided by objective physicochemical and biochemical factors, primarily electric charge, hydrophobic characteristics and steric interactions, the most abundant proteins present in the biological environment or matrix, such as albumin in plasma or serum, may often be [175], but not necessarily, the primary contributor to the protein corona [222]. The dynamic evolution in protein corona function also implies that the composition, structure, shape of the protein corona, even its hard core, can be substantially different during *in vitro* studies compared to "real-life" therapeutic administration [243]. The effect of the protein environment and the dynamic characteristics of the protein corona complicate the development of suitable truly smart therapeutic nanoformulations (a) capable to avoid unwanted cellular uptake, (b) pass through targeted biological barriers, and (c) target the diseased site. In addition, the immunological status of patients may affect the protein corona; as an example pre-existence of circulating anti-PEG IgM and their binding on PEGylated NPs such as liposomes alters the composition of the protein corona through activation of the complement system, leading to altered physiological pathways and decreased circulation times [64].

7.3. Composition of protein corona of nanoformulations around BBB

A recent study has revealed quantitative and qualitative evolution of the protein corona surrounding gold NPs coated with 11-mercapto-1-undecanesulfonate once passing through the BBB [163]. This study demonstrated that the protein corona formed on engineered NPs was dramatically affected, underwent quantitative/qualitative molecular modifications by interaction with the BBB and was more stable once it has crossed the barrier. Thus, the initial composition of NP protein corona was not predictive of particle's fate and performance once beyond the barrier at the target organ. This alteration may affect the targeting efficiency of brain (glioma)-targeted delivery system. To perform their evaluation, the authors used an *in vitro* cellular model of BBB where a monolayer of cerebral microvascular endothelial cells was grown on a Transwell system. The apical compartment containing a grown medium with 5% serum was designed to mimic the "blood side", whereas the basolateral compartment containing a serum-free growth medium represented the "brain side". Controls were performed to verify that maximum tight function formation was achieved. The NPs were seeded in the apical compartment and recovered in the basolateral compartment. Proteomics studies of the protein corona revealed striking changes occurring during the crossing of the endothelium cell layer over 3 hours. Indeed from 20 abundant proteins present in the protein corona in the "blood compartment", only 9 could be detectable when the NPs reached the "brain compartment", and several other proteins, not derived from the endothelial cells, were enriched [163].

However, such *in vitro* models, based on optimal preservation of cellular tight junctions, have limitations to predict the transport of nanoformulations though the BBB of patients with neurological disorders, as neurodegeneration can be associated with BBB breakdown or alterations of molecule transport. Hence, further experiments based on an alternative *in vitro* BBB models or on *in vivo* experiments involving the collection of NPs in the CSF after systemic administration are needed. This will help to better understand the dynamic evolution in the protein corona on various types of nanoformulations and their impact on the recovery in the brain interstitial fluid or the CSF. Also, the development of novel *in vitro* BBB models using induced pluripotent stem cells (iPSCs) isolated from patients with neurodegenerative diseases is expected to become instrumental in the development of more effective therapeutic strategies and nanoformulation systems [185]. It should also be noted that nanoformulations once internalized by endothelial BBB cells and penetrated into the brain, could be coated with different types of biomolecules (protein corona) depending on the type of biological fluid they are exposed to during their *in vivo* journey after the administration. Nanocarriers' surface properties may thus exert diverse unexpected effects. Such effects may produce off-target effects of brain-targeted nanoformulations as the protein corona may diminish the specific reaction between a ligand and the targeted receptor. For example, using transferrin-conjugated NPs, proteins in the biological media shielded transferrin from binding to its targeted transferrin receptors due to NPs interaction with other proteins and protein corona formation in the medium [244]. Since this effect has not been investigated much, further evaluation using *in vitro* or *in vivo* experiments is needed.

7.4. Nanoformulation properties affecting the protein adsorption and corona formation

The size and charge of the NPs are important factors influencing protein binding, conformational changes, and composition of protein corona. However, other factors such as shape, composition, morphology, and hydrophobicity of NPs also affect the protein adsorption. NPs larger than proteins (which have a mean apparent size of 5-12 nm depending upon their molecular mass) lead to protein stretching and possible structure alterations, whereas proteins larger than NPs have looser interactions and fewer risks of denaturation [245]. A detailed study of *in vivo* protein corona formation after blood circulation of anisotropic gold NPs of different shape and size (nanorods and nanostars), coated with PEG, was performed. Results revealed that the total amount of protein adsorbed onto the particle surface and the protein corona composition was affected by both the particle size and shape [246]. The toxicity of NPs (graphene oxide nanosheets) was studied by allowing them to physically damage the cell membrane. The toxicity was decreased with an increase in the fetal calf serum concentration in the culture medium. This was explained by a high protein adsorption capacity of NPs, which covered the NP surface, thereby changing the shape of the NPs and partly preventing the cell membranes damage [247].

The effect of gold NPs morphology on the structure and function of adsorbed enzymes, lysozyme (Lyz) and α-chymotrypsin (ChT), has been investigated. Gold nanospheres (AuNS, diameters 10.6 ± 1 nm), and gold nanorods (AuNR, dimensions of $10.3 \pm 2 \times 36.4 \pm 9$ nm) were synthesized. Under saturating conditions, proteins adsorb with a higher surface density on AuNR compared to AuNS. The adsorption of Lyz on AuNS and AuNR resulted in a 10% and 15% loss of secondary structure, respectively, led to aggregation and greatly reduced enzymatic activity. The secondary structure and activity of ChT on AuNS and AuNR was retained at low surface coverages, however, as protein loading approached monolayer conditions on AuNR, a 40% loss in secondary structure and 86% loss of activity were observed. From the results, it was clear that AuNP morphology didn't affect the structure of the adsorbed protein [248].

The secondary structure of proteins is strongly affected by the surface charge of NPs [151]. For instance, components from human plasma contributing quantitatively to the protein corona of silica NPs include Apo B100, complement factor H, fibronectin, factor V, and others, with variations depending upon the charge (bare silica, with NH_2 group and with COOH group) of the NPs [151]. Colloidal gold NPs were modified with amphiphilic polymers to obtain NPs with identical physical properties except for the sign of the charge (negative/positive) to study the charge-dependent interactions of NPs with biological media and cell uptake. Results showed that the number of adsorbed human serum albumin molecules per NP was not influenced by their surface charge. Positively charged NPs were taken by cells to a larger extent, both in serum-free and serum-containing media. Consequently, with and without protein corona, NP internalization was dependent on the sign of charge. The uptake rate of NPs by cells was higher for positively than for negatively charged NPs. Furthermore, cytotoxicity assays revealed higher cytotoxicity for positively charged NPs, associated with their enhanced uptake [249]. Hydrophobic surface NPs adsorb more protein, thus, thus may cause agglomeration and opsonized more quickly than hydrophilic NPs, leading to shorter systemic circulation time in blood [250]. One study investigated the changes in the plasma protein adsorption patterns of latex NPs due to surface hydrophobicity variation. Correlations between physicochemical characteristics and the protein adsorption patterns have been found [251].

7.5. Effect of experimental conditions on protein corona formation

Protein adsorption can also be significantly affected by experimental conditions and associated factors such as temperature, pH, exposure time, protein concentration, etc. The effect of temperature variation on the formation and composition of protein corona was studied on a set of NPs with various surface chemistries and electric charges [252]. The results concluded that the degree of protein coverage and the composition of the adsorbed proteins on the NP's surface, including the cellular uptake were influenced by temperature and have a great impact on the cellular uptake as well. Therefore, the temperature needs to be monitored while administering nanomedicine products to the patients. In addition to temperature, changes in pH can also alter NP protein corona composition [253]. Moreover, proteins molecules have a defined conformation and carry a net surface charge depending on the pH of the environment (medium) they are present. Protein corona is also

significantly affected by the protein concentration in the media. Polyethyleneimine coated magnetic NPs (MNPs) were incubated in a medium containing a varied composition of fetal calf serum (FCS). Zeta potential, as well as SDS-PAGE observations, revealed an increase in the amount of proteins on MNPs corona with an increasing amount of FCS in the medium [254].

7.6. Impact of biological milieu on protein corona

There is scientific evidence that the composition of the protein corona is, as expected, greatly influenced by the biological medium in which NPs are suspended. An increasing number of studies have therefore tried to (a) understand the binding mechanisms of proteins on NPs, (b) elucidate the role of the protein corona formed upon addition to body fluids on NPs *in vivo* fate, as well as (b) scrutinize and model how pre-treatment by proteins could be useful at governing NPs toxicity and targeting [151]. We introduce below findings on the impact of different protein fluids on the composition of the protein corona.

7.6.1. Fetal bovine serum (FBS)

Understandably a majority of *in vitro* studies have focused on protein corona formation when exposing NPs to FBS, the main experimental supplement of growth media used so far. It is clear that FBS can dramatically affect the interaction between NPs and cells. The presence of an FBS-generated protein corona on silica NPs lowers their adhesion to the cell membrane and decreases the internalization [255]. *In vitro* experiments have shown that the concentration of FBS can have an impact on the apparent toxicity of some NPs. For instance, when graphene oxide nanosheets are incubated with increasing doses of FBS there was a parallel decrease in cellular toxicity due to the formation of the protein corona that protects cells from the physical damages induced by "naïve" graphene oxide [247]. Use of heat-inactivated FBS, to inactivate the complement, impacts NP protein binding and cellular uptake, compared to medium supplemented with untreated FBS [169]. The nature of the basal medium itself (DMEM versus RPMI) can influence the amount and range of FBS proteins adsorbed on 15 nm gold NPs as well as the extent of internalization by cells [256]. Divalent ions and macromolecules in FBS-supplemented growth media influence the protein corona of polyacrylic acid-coated cobalt ferrite NPs and silica NPs, with differences in the number and nature of complement- and immune system-related proteins [167]. Cell cultures performed under flow lead to a higher content of FBS-derived protein in the corona of polystyrene NPs, with higher plasminogen content, and an associated decreased cellular binding [239]. In serum-free medium, naked silica NPs get coated by cell-derived proteins with a differential impact on cellular uptake compared to FBS-coated NPs [255]. The formation of the protein corona by the membrane or cytosol proteins can take place within an hour of NPs administration [151].

Impact of FBS on protein corona depends upon NP formulation. Several studies have evaluated the impact of chemical nature, size, and curvature of NPs on protein corona formed in FBS medium. The fine chemistry of native or modified silica NP has an impact on the final composition of the serum protein corona, with influences also on NP agglomeration kinetics and toxicity on alveolar macrophages and mouse lung epithelial cells [257]. The protein corona of 3.5-150 nm citrate-stabilized gold NPs (AuNPs) exposed to FBS-supplemented medium evolves dynamically towards the formation of the hard corona, the phenomenon being faster as NP size decreases [258]. Silica mesoporous NPs of small size (70 nm) and higher curvature exposed to FBS exhibit a stronger relative capacity to adsorb proteins, especially those with a molecular mass less than 50 kDa, compared to larger NP (900 nm). NP porosity leads to preferential adsorption of small FBS proteins due to a size exclusion phenomenon [232]. Silver NPs (AgNPs), including citrate-stabilized and polyvinlypyrrolidone-(PVP)-stabilized colloidal silver of different size (20 or 110 nm) and curvature, share a common capacity to adsorb a range of FBS proteins, in particular albumin and Apo(s). Citrate- and PVP-stabilized AgNPs (size: 110 nm) bound the highest number of proteins compared to 20-nm citrate- and PVP-stabilized AgNPs, suggesting an impact of surface curvature. More hydrophobic proteins were found on the 20-nm AgNPs compared to 110 nm counterparts [259]. These data confirm that extensive experimental work is needed to understand NP and FBS interactions leading to protein corona formation.

7.6.2. Plasma

Predictive studies of the influence of the protein corona on NPs intended for intravenous administration should preferentially be done using human plasma, rather than FBS. Several studies evidence a strong binding of plasma proteins on various NPs *in vitro.* The plasma-protein corona increases the hydrodynamic diameters of polymeric NPs and induces a general shift toward moderately negative surface charges [260], associated with decreased uptake of colloidal NPs by primary human keratinocytes and human umbilical vein endothelial cells but no significant change for nanogels. Protein corona of nanogold NP stimulated cytokine release from primary human macrophages but not the colloidal NPs. The protein corona can also in some situation induce a decrease of the drug released by solid cationic NPs [260]. A systematic study of the protein corona around 20 nm modified silver and gold NPs with various surface chemistries confirmed a switch towards negative charges after contact with plasma [261]. Among 300 proteins found in the corona, 99 were common to each NP, albumin and Ig(s) representing only about 2%. Factors contributing to protein corona formation are the surface charge and core of the NPs and the isoelectric point of the plasma proteins. Plasma fibrinogen, which is depleted in serum, preferably binds to negatively charged gold NPs [261]. In *in vitro* studies plasma proteins, apart albumin, exhibited very low binding affinity for biocompatible amphiphilic copolymers; no hard corona was detected around N-(2-hydroxypropyl)methacrylamide-based NP, confirming the impact of NP chemistry on plasma protein corona [225]. The protein corona, made of various proteins, formed during incubation of albumin particles in human plasma, decreases the binding to HUVEC cell membranes and lowers initial cellular uptake [262].

Interestingly, PEG binding on liposomes engineered to treat metastatic pancreatic ductal adenocarcinoma does not prevent plasma protein adsorption, as plasma protein corona binding is influenced more by the liposome surface chemistry than by PEG; this protein corona was found to enhance the cellular uptake by the PANC-1 cell line [263]. A positive impact of the protein corona is not universal, though. Indeed, most of the plasma proteins that contribute to the corona of black phosphorus (BP) quantum dots and nanosheets are immunologically active, increasing macrophage uptake, exerting immunotoxicity and immune perturbation associated with NF-kappaB pathway activation and a significant increase in cytokine secretion [264]. *In vitro* experiments using plasma-generated corona can help in designing NP with better targeting efficiency, but, may not completely mimic what eventually occurs in the blood environment. For example, the targeting efficiency of NPs modified by transferrin receptor-ligands of different size and by transferrin is lost in a plasma medium *in vitro*, but still partially retained after *in vivo* corona formation. The phenomenon and cellular impact (internalization and exocytosis) were influenced by ligand size as well as conformation and source of the corona [265].

The species used to generate plasma is also influential. It is relevant to do protein corona experiments using the plasma of the same animal species used as an experimental model, in addition to human plasma: the extent of NP aggregation and protein corona composition may indeed vary depending upon the plasma source [266]. For instance, the protein corona around 25-nm silicon dioxide NPs (SiO_2 NPs) functionalized with PEG and transferrin contains mostly Ig(s), actin cytoplasmic 1, hemoglobin subunit beta, serotransferrin, ficolin-3, complement C3, and Apo A-1 when using human plasma, and serine protease inhibitor A3K, serotransferrin, alpha-1-antitrypsin 1-2, hemoglobin subunit beta, and fibrinogen gamma and beta chains when using mouse plasma.

Table 3
Anticoagulants used for the collection of blood and preparation of plasma.

Anticoagulant type	Composition	Ratio
ACD-A [302]	Sodium citrate dihydrate 22.0 g/l Citric Acid Hydrous 8.0 g/l Dextrose monohydrate 25.38 g/l pH (25°C) 4.7 - 5.3	15/100 mL of blood
ACD-B [302]	Sodium citrate dihydrate 13.2 g/l Citric Acid Hydrous 8.0 g/l Dextrose monohydrate 15.18 g/l pH (25°C) 4.7 - 5.3	25/100 mL of blood
CPD [302]	Sodium citrate dihydrate 26.3 g/l Citric Acid Hydrous 3.7 g/l Dextrose monohydrate 25.5 g/l Sodium Biphosphate 2.22 g/l Sodium hydroxide 1N (pH adjustment) pH (25°C) 5.3- 5.9	14/100 mL of blood
CPD-A [302]	Sodium citrate dihydrate 26.3 g/l Citric Acid Hydrous 2.99 g/l Dextrose monohydrate 29 g/l Sodium Biphosphate 2.22 g/l Adenine 0.27 g/l Sodium hydroxide 1N (pH adjustment) pH (25°C) 5.3- 5.9	14/100 mL of blood
CP2D [302]	Sodium citrate dihydrate 26.3 g/l Citric Acid Hydrous 3.7 g/l Dextrose monohydrate 50.95 g/l Sodium Biphosphate 2.22 g/l Sodium hydroxide 1N (pH adjustment) pH (25°C) 5.3- 5.9	14/100 mL of blood
4% citrate [302][a]	Sodium citrate dihydrate 40 g/l Citric Acid Hydrous: as required for pH adjustment pH (25°C) 6.4 - 7.5	6.25/100 mL of plasma
EDTA [311]	Salt of ethylene diamine tetraacetic acid. Dipotassium (K2), tripotassium (K3) (41) and disodium (Na2) salts are used; concentrations:	1.2 to 2.0 mg/mL blood (4.1 to 6.8 mmol/L blood) based on anhydrous EDTA.
Heparin [311]	Unfractionated sodium, lithium or ammonium salt of heparin with a molecular mass of 3 to 30 kD	12 to 30 IU/mL of blood

[a] Used for plasma collected by apheresis.

Such differences can lead to distinct biological responses [267]. The impact of lipoproteins in plasma, as contributors to a "lipoprotein corona" also deserves more investigations. Lipoproteins in contact with polymeric NPs, disintegrate, leading to protein adsorption and a major effect on cell uptake [236]. In addition, the impact of the anticoagulant used during plasma preparation even should not be neglected (Table 3): a citrated anticoagulant solution was selected in studies where heparin impacted the fate of cellular uptake by NPs [179].

7.6.3. Human serum

From a translational viewpoint, and as was observed with plasma [266], use of human serum in place of FBS for cell experiments intended to study NP impact on viability, toxicity, or cellular uptake is logical, especially when evaluation involves human cells. In addition, preferring human serum is justified when human plasma or native platelet lysates may induce medium gelation in the absence of heparin [181], an anticoagulant that may interfere with cell properties. In studies with vascular-targeted NPs, use of serum in place of anticoagulated plasma has been found superior to assess the drug carrier adhesion [268].

7.6.4. Albumin

Since albumin is the most abundant protein in plasma and serum, although not always the one contributing most to the protein corona, several studies have evaluated it as a model protein, or as an experimental tool to evaluate the impact of pre-coating of NP on protein structure and NP cellular uptake or toxicity [269,270]. Pre-coating of branched gold NPs by human serum albumin (as well as human plasma) can differentially modulate the uptake by liver cells and therefore toxicity [270]. Binding of albumin on gold NPs with different surface charges is similar but, compared to negatively charged NPs, those with a positive charge associated more with cells and internalized faster, potentially by stronger interaction with scavenger receptors [151,161]. Functionalized polystyrene NPs with cationic or anionic surface pre-exposed to serum albumin also target different cell receptors resulting in different functionality. This has been attributed to change in the conformation of albumin in contact with cationic NPs [161]. BSA influences gelatin-oleic NPs (GONs) physicochemical properties by increasing the size, slightly reducing the negative net charge, and improving stability. A pre-coating by BSA was found to modulate, positively or negatively, *in vitro,* the uptake by human embryonic kidney cells 293 (HEK 293) and human adenocarcinoma alveolar basal epithelial cells (A549), offering a possible strategy for higher efficacy and targeting *in vivo* [271].

7.6.5. Other biological fluids or proteins

Synthetic carbonate-apatite NPs in contact with various body fluids, including serum, CSF, and synovial fluid, developed a protein corona reflecting the protein composition of the biological milieu. Interestingly, as determined by LC-MS/MS, a few proteins were commonly adsorbed in all conditions evaluated: albumin, fetuin-A, complement C3, alpha-1-antitrypsin, prothrombin, and Apo(s) A1 and B-100 [272]. Future studies should evaluate in great details the impact of body fluids such as tears, CSF, synovial fluid on the protein corona of various NPs as a means to better realize their fate in such biological milieu, since much experimental information is missing.

Various factors encompassing the physicochemical properties of nanoformulation, the experimental conditions used and the nature of the biological milieu, which can affect the protein corona formation of nanocarriers, are summarized in Fig. 6.

7.7. Forming artificial protein corona on nanoformulations for efficient brain-targeting drug delivery approaches

The formation of a protein corona due to non-specific interactions between nanocarriers and plasma proteins upon systemic administration affects the *in vivo* fate and biological responses of nanocarriers. In this regard, purposely pre-forming a corona through surface modification of nanoformulations by proteins, using incubation or covalent conjugation approaches, may modulate the *in vivo* plasma proteins adsorption and enhance the targeting ability, ultimately prolonging the blood circulation time of nanoformulations and efficiently enhancing drug delivery to the brain [273,274]. Using this approach, recently, lipid NPs have been functionalized before their intravenous administration by incubation with ApoE4 protein to increase the amount of drug delivered to the brain [275]. The research design was based on the fact that ApoE has played a significant role in the delivery of NP-based drugs across the BBB due to its preferential binding to specific receptors on the BBB [276,277]. Results showed that the adsorption of ApoE4 onto polysorbate 80-stabilized lipid NPs produced an artificial protein corona, which increased NP translocation into brain parenchyma and improved brain accumulation by 3-fold compared to undecorated NPs. These findings support the concept that artificially-decorated protein corona on nanocarriers has high clinical translational potential and may improve the development of nanotechnology-based therapeutics for neurological diseases. However, one potential drawback to this artificial protein corona strategy is the weak interactions between the protein and the NP surfaces. Hence, further studies are needed to characterize the stability of the artificial protein corona especially in *in vivo* conditions to understand how the protein corona is affected by the shear stress of the systemic circulation and the presence of plasma proteins. In addition, when such proteins are combined with

Table 4
Analytical methods for the evaluation of physicochemical properties of nanoformulations [312–316].

Techniques	Working principle	Characteristics analyzed	Strengths	Limitations
Dynamic light scattering (DLS) or Photon Correlation Spectroscopy (PCS) or Quasi-elastic light scattering (QELS)	• Measures variation in scattered intensity with time at a fixed scattering angle • The Brownian motion of particles in suspension causes laser light to be scattered at different intensities • Analysis of these intensity fluctuations yields the velocity of the Brownian motion and hence the particle size is analyzed using the Stokes-Einstein relationship	• Hydrodynamic size • Size distribution	• Non-destructive and non-invasive • Rapid and simple • Reproducible measurement • Measurement can be performed in any liquid media, solvent • Provides accurate measurements for monodisperse samples • Cost-effective • Broad concentration range of sample analysis • Measures particle size across the range ~ 0.1nm to ~ 10μm • Sample volumes are small in microliters range. • High throughput analysis with high scalability potential	• Significant influence of small numbers of large particles in the sample • Limited in measuring the polydisperse samples • Limited size resolution • Assumption of spherical shape samples, however, not all particles are spherical in shape • Measures the whole population together and give a single result • Limitations in measuring turbid, translucent or transparent samples • Cannot distinguish between individual particles and aggregates • Particle has to be in suspension form and undergoing Brownian motion • Requires solvent refractive index • Light absorption by the dispersant can interfere with detection
Static light scattering (SLS)	• Measures scattered intensity as a function of angle	• Hydrodynamic size • Size distribution • Shape • Morphology	• Non-destructive and non-invasive • Rapid and simple • Reproducible method • Measurement can be performed in any liquid media, solvent • Broad concentration range of sample analysis	• Significant influence of small numbers of large particles in the sample • Limited in measuring the polydispersed samples • Applicable only for monomodel size distribution • Measure the whole population together and give a single result • Limitations in measuring turbid, translucent or transparent samples
Nanoparticle tracking analysis (NTA)	• Utilizes the properties of both light scattering and Brownian motion	• Size • Size distribution • Concentration	• Individual particle size distribution • Monomodel and multimodel size distribution • Avoids the influence of few large particles in the sample • Unbiased high peak resolution for polydisperse samples • Provides visual confirmation and direct observation of particles in sample • High resolution • High throughput analysis with high scalability potential • Concentration of the sample can be measured (lieally in the range of 10^7 to 10^9 particles/ml) • Possibility to determine the size of fluorescent samples • Requires no information about solvent refractive index. • Ability to measure size and aggregation state changes in the presence of serum proteins • Better suited for polydisperse samples than DLS, since particles are visualized individually	• Time consuming • Good for only diluted samples • Narrow concentration range analysis • Expensive measurement • Lack of precise and repeatable measurement • Electro-osmotic effect • The size and concentration of particles with similar Brownian motion cannot be distinguished • The accuracy of the determination of the concentration of samples is strongly affected by their size and instrument parameter settings • Operates for particles only from about 10 to 1000 nm in diameter • Requires operational skills to learn software settings and instrumental parameters
Field flow fractionation (FFF) coupled DLS-multi angle light scattering (MALS)	• FFF is a flow based separation method • In MALS, a polarized single frequency light beam is focused on sample or particle and the scattered light is detected with multiple detectors positioned at various angles	• Size • Size distribution • Molecular weight • NPs-protein interaction	• Provide size information for samples in physiological media • Measure change in nanoparticle size distribution after protein binding • Able to discriminate between dimers and larger particles by their shape	• Complex algorithm to extract size distribution • Particles in agglomerates or aggregates are not determined • For good separation performance, choice of buffer, liquid flow, membrane geometry, have to be carefully optimized • A standard should be used to normalize the MALS each time a solvent is changed
Optical microscopy	• An objective lens with very short focal length is used to form a highly magnified real image of the object	• Size distribution of nanomaterials	• Rapid and simple • No specific sample preparation required	• Low resolution limit • Unable to provide comprehensive information

(continued on next page)

Table 4 (*continued*)

Techniques	Working principle	Characteristics analyzed	Strengths	Limitations
		especially of aggregates	• Easy to be integrated with digital camera systems	
Scanning electron microscopy (SEM)	• Bombards a sample with a stream of electrons and monitors the resulting scattering effects	• Size and size distribution • Shape • Aggregation	• Direct measurement, rapid and and high resolution technique • Simulation of 3D structure offer better performance for surface and shape analysis than TEM • Allows visualization of small particles under very high magnification • Provides visual confirmation • Enables a larger amount of sample to be measured at one time, which can improve both the statistical reliability and efficiency of nanoparticle size and shape distribution measurements	• Conducting sample or coating conductive materials required • Sample analysis in non-physiological conditions (dry samples required • Biased statistics of size distribution in heterogeneous samples • Expensive equipment and slow measurement process • Special training is required to operate an SEM as well as the process of sample preparation • Cryogenic method required for most NP-bioconjugates • Low throughput and scalability • Low statistical robustness
Environmental SEM (ESEM)	• Bombards a sample with a stream of electrons and monitors the resulting scattering effects	• Size • Size distribution • Shape • Aggregation	• Direct measurement of the size/size distribution and shape of nanomaterials • Sample analysis in physiological conditions • Does not require the use of fixing, staining or freezing of samples • Rapid observation • Allows visualization of small particles under very high magnification • Allows imaging of dynamic changes of wet systems without previous sample preparation • Able to modify sample environment, including pressure, temperature and gas compositions	• Conducting sample or coating conductive materials required • Biased statistics of size distribution in heterogeneous samples • Expensive equipment • Cryogenic method required for most nanoparticle-bioconjugates • Low resolution • Enables a larger amount of sample to be measured at one time, which can improve both the statistical reliability and efficiency of nanoparticle size and shape distribution measurements • Cannot provide detailed information regarding the lamellarity and internal architecture of the nanoscale structures
Scanning tunneling microscopy (STM)	• Based on the concept of quantum tunneling	• Size • Shape • Roughness • Dispersion • Aggregation • Surface defects	• Direct measurement • High spatial resolution enable surface exanimation at an atomic level • Gives 3D profile of a surface, which allows examine a multitude of characteristics • Characterizes electrical and photonic properties of single particles • Versatile technique that can be used in ultra-high vacuum, air, water and other liquids and gasses • Can be operated at very low and high temperatures	• Conductive surface required • Surface electronic structure and surface topography unnecessarily having a simple connection • Projection limitation and that there is no depth sensitivity in a single image • Use highly specialized equipment that is fragile and expensive
Transmission electron microscopy (TEM)	• Transmission of electron beams through a sample and creation of an image	• Size • Size distribution • Shape heterogeneity • Aggregation • Dispersion	• Direct measurement of the size/size distribution and shape of nanomaterials with higher spatial resolution than SEM • Several analytical methods can be coupled with TEM for investigation of electronic structure and chemical composition of nanomaterials • Direct visualization and observation of structure and surface morphology	• Ultrathin samples is required • Samples in non-physiological condition • Sample damage/alternation • Potential artifacts can be generated by complex sample preparation method • Expensive equipment • Whole system must be under a vacuum • The extreme magnifications make it difficult to obtain a truly representative sample, which is why it is important to pair TEM with a correlative technique whenever possible • It is sometimes difficult to even locate the NP of interest when examining features on the scale of cells, organs, or organisms • Staining often applied to increase the imaging contrast may damage particles and affect their morphology • Low throughput capacity and scalability

Table 4 (*continued*)

Techniques	Working principle	Characteristics analyzed	Strengths	Limitations
Atomic force microscopy (AFM)	• Based on cantilever/tip assembly that interacts with the sample	• Size and size distribution • Shape • Sorption • Dispersion • Aggregation • Surface properties • Morphology (Topology)	• Generates high-resolution 3D surface images • Sub-nanoscaled topographic resolution • Direct measurement of samples in dry, aqueous or ambient environment (does not require a vacuum environment) • No need of sample labelling and conductivity • Can provide information about the mechanical and chemical properties of a sample surface through force measurements • Capable of characterizing dynamics between nanomaterials in biological situations	• Needs substantial user training and experience • Requires nanoparticles to be adsorbed onto support surfaces, which can modify the size and shape of the vesicles • The size of the cantilever tip is generally larger than the dimensions of the nanomaterials examined, leading to unfavorable overestimation of the lateral dimensions of the samples • Poor sampling and time consuming • Limitation of observing only particle surface properties • Slow in scanning an image • Lacks the capability of detecting or locating specific molecules (but eliminated by recent progress in single--molecule force spectroscopy with an AFM cantilever tip carrying a ligand, a cell adhesion molecule or chemical groups, which can detect single functional molecules on cell surfaces) • Low throughput capacity and scalability
Near-field scanning optical microscopy (NSOM)	• Near-field imaging occurs when a sub-micron optical probe is positioned a very short distance from the sample and light is transmitted through a small aperture at the tip of this probe	• Shape • Size	• Concurrent measurement of fluorescence and spectroscopy • Simultaneous measurements of the topography and optical properties (fluorescence) • Nano-scaled surface analysis at ambient conditions • Assessment of chemical information and interactions at nanoscaled resolution • High-resolution topographic information of nanomaterials • Can analyze the distribution of single molecules on the surfaces of cells and interactions in protein–NP conjugates	• Long scanning time for high resolution images or large specimen areas • Incident light intensity is deficient to stimulate weak fluorescent molecules • Difficulty in imaging of soft materials • Analysis limited to the nanomaterial surface as small specimen area are analyzed • Only surface features can be imaged and studied
Electrophoretic light scattering (ELS) (Zeta potential)	• Based on laser Doppler electrophoresis, where the charged particles in the presence of an electric field, migrate towards the oppositely charged electrode and are illuminated with a laser light beam	• Surface charge • Stability	• Concurrent measurement of several particles • Minimal sample preparation is needed • Very low sample volume is required • Cost effective measurement	• Measurement dependent on the type of dispersant/solvent • Not suitable for samples heterogeneous in size (light scattered by larger NPs may mask that of smaller NPs, resulting in the measured z-potential being affected by a sub-population of the NPs) • Not suitable for samples made of a range of materials with different optical properties which cause them to scatter light more or less effectively • Electro-osmotic effect • Lack of precise and repeatable measurement
Tunable resistive pulse sensing (TRPS)	• Based on the Coulter principle and measures the reduction in ionic current across a pore on a membrane due to the temporary occlusion as a particle traverses it	• Size • Concentration • Zeta potential	• Simultaneously measures size and zeta potential on a particle by particle basis • Direct measure of particle concentration, and high resolution analysis of particle size and surface charge • Tunable pore size increases the dynamic range of TRPS, making it suitable for analysis of extremely polydisperse samples • Measurements are independent of the material composition of the particles, and therefore do not suffer from the same loss of sensitivity as optical techniques • High-throughput (~3000 particles/-min) single particle analysis • Large dynamic size and concentration	• Limitations to how much each pore can be stretched, thus needs to match a membrane with a pore size to the sample of interest • May not be able to discriminate between different types of particles

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Table 4 (*continued*)

Techniques	Working principle	Characteristics analyzed	Strengths	Limitations
			range analysis • High precision concentration analysis • Very small sample volume	
Fluorescence correlation spectroscopy (FCS)	• Correlation analysis of fluctuation of the fluorescence intensity	• Hydrodynamic dimension • Size • Shape • Concentration • Binding kinetics	• High spatial and temporal resolution • Can document chemical kinetics, molecular diffusion, concentration effect, and conformation dynamics • Low sample consumption • Specificity for fluorescent probes • Can quantify the real time mobility of fluorescently labelled receptors or ligands within regions of single living cells • Small sample volumes (< 10 μl) and concentrations (picomolar) are required • Short analysis time • *In situ*, nondestructive and largely non-perturbing technique	• Limited applications and inaccuracy due to lack of appropriate interpreting models
• Raman scattering (RS) • Surface enhanced Raman (SERS) • Tip-enhanced Raman spectroscopy (TERS)	• Measures the inelastic scattering of photons possessing different frequencies from the incident light after interacting with electric dipoles of the molecule	• Hydrodynamic size and size distribution (indirect analysis) • Conformation change of protein–metallic NP conjugate • Structural, chemical and electronic properties	• Non-destructive and non-invasive technique • Suitable for studying biological samples in aqueous solution • Works in-situ and in-vitro for biological samples • Works under a wide range of conditions (temperature, pressure), no vacuum needed • Potential of detecting tissue abnormalities • SERS provides enhanced RS signal and increased spatial resolution • SERS and TERS provide topological information of nanomaterials • SERS can be used to study surface functionalization of NPs, monitor the conformational change in proteins conjugated to the NPs, and track intracellular drug release from the nanocarriers • SERS detects very low concentrations whereas TERS resolve very small features of materials • High throughput and scalability	• Relatively weak single compared to Rayleigh scattering • Limited spatial resolution (only to micrometers) • Extremely small cross section • Interference of fluorescence from other materials present in the sample • Irreproducible measurement of SERS caused by the size and shape variation, as well as undesirable aggregation of NPs • Low sensitivity due to weak Raman signals and strong fluorescence due to impurities.
X-ray diffraction (XRD)	• Uses X-rays to obtain structural information about the sample	• Size • Shape • Composition • Polymorphism	• Well-established method • High spatial resolution • Non-destructive • Easy sample preparation • Determine the material structure at the atomic scale	• Limited applications in crystalline materials only • Not suitable for amorphous materials and the peaks are too broad for particles with a size below 3 nm • Only one conformation or binding site for sample accessible • Low intensity of diffracted X-rays compared to electron diffraction • More accurate for measuring large crystalline structures. Small structures present in trace amounts often go undetected which can result in skewed results
Small-angle X-ray scattering (SAXS)	• Uses X-rays to obtain structural information about the sample	• Size • Size-distribution • Shape • Growth kinetics • Location of nanostructures	• Information by examining either crystalline or amorphous materials • Non-destructive, with simplified sample preparation • Amorphous materials and sample in solution accessible • Biological specimens can potentially be studied in their natural environment • Fast	• Relatively low resolution but recent progress in SAXS can achieve higher resolution measurements by introducing synchrotron as the high--energy X-ray source • High background noise • Large differences in the scattering signal of particles with different sizes, leading to uncertain size distributions in samples containing polydisperse particles • Specialized infrastructure and trained personnel required • High instrument cost

nanocarriers *ex vivo*, as a component of a nanoformulation, they should be approved for clinical applications, which restrict the range of therapeutic proteins available for such purposes.

8. Exploiting cells infiltration across the BBB as drug targeting approaches to brain

Living cells as drug carriers ha ve opened new therapeutic avenues for various indications including CNS diseases [86,278–280]. Cells such as macrophages, leukocytes, neutrophils and monocytes are naturally capable of crossing the BBB and are ideal cellular vehicles for drug delivery to the brain [281,282]. Recently, cellular "backpacks" loaded with an antioxidant, catalase, were attached to autologous macrophages and systemically administered into mice affected by brain inflammation [283]. The results indicated that engaging natural immune cells as drug carriers is a novel strategy for the treatment of neurodegenerative diseases. Macrophages were also exploited as 'Trojan horses' to carry drug-loading NPs, pass through barriers, and offload them into brain tumor sites [284]. In this approach, anticancer drugs loaded NPs were incubated with RAW264.7 cells to prepare macrophage-NPs (M-NPs). The improvement of cellular uptake and penetration into the core of glioma spheroids of M-NPs compared with NPs was confirmed in *in vitro* studies. The tumor-targeting efficiency of M-NPs was observed to be significantly enhanced in nude mice.

Generally, NPs BBB-crossing using cells could be achieved either by embedding NPs into activated cells as 'Trojan horses' approach to reach the brain, or by designing cell-mimicking NPs to avoid their clearance by the immune system, thus providing longer circulation in blood and enhanced brain targeting efficiency. In the first approach, after phagocytosis of NPs, immune cells transport their cargo into the brain. For example, the cyclic RGD (cRGD) peptide-modified liposomes (cRGDL) showed high affinity for monocytes *in vitro* and *in vivo* and co-migrated across *in vitro* BBB model [285]. Using the second approach, nanoporous silicon particles successfully evaded the immune system, crossed the biological barriers and localized at target tissues when they are coated with cellular membranes purified from leukocytes [286] In spite of the fact that the investigations in these directions are limited, these approaches of using natural immune cells as drug carriers can potentially provide more effective treatment of brain-associated disorders especially when they are associated with increased infiltration of immune cells across the BBB.

Circulatory cells such as RBCs, platelets, leukocyte, etc., have received a significant interest as DDS because of several distinctive features arising from their unique structures, mechanical properties and natural capabilities to cross the biological barriers [86]. Particularly RBCs have been much explored to act as an efficient and safe DDS because of several attractive features. RBCs highly flexible/elastic nature enables these cells passing through narrow capillaries and organs such as spleen and liver. Several studies inspired by RBCs elasticity, shape, and diameter, developed synthetic RBCs (sRBCs) to generate long-circulating drug carriers [287–289]. Because of these attributes, sRBCs were also applied in brain-targeted drug delivery approaches. For example, an effective brain-targeted DDS was developed that combined a robust RBC membrane-coated NP (RBCNP) with a unique neurotoxin-derived targeting moiety [290]. The RBCNPs retained the complex biological functions of natural cell membranes while exhibiting physicochemical properties that are suitable for effective drug delivery. DCDX peptide was successfully incorporated onto the surface of RBCNPs without compromising the peptide's brain targeting ability both *in vitro* and *in vivo*. The DCDX-RBCNPs loaded with doxorubicin had superior therapeutic efficacy and reduced toxicity compared to the non-targeted formulations, demonstrated in a glioma mouse model. In another recent study, RBC-hitchhiking (nanocarriers adsorbed onto the RBCs) was applied to improve the delivery for a wide range of nanocarriers to selected organs, including the brain [291]. Results confirmed that the intra-carotid artery injection of RBC-hitchhiking nanocarrier delivered >10% of the injected nanocarriers dose to the brain, ~10× higher than that achieved with affinity moieties.

In summary, immune/circulatory cell-based drug delivery approaches to brain are novel and hold promises in improving drug therapeutic effects; however, there are some challenges as well. Natural cell carriers are susceptible to contamination during the *ex vivo* or *in vitro* processing in formulation development that can produce side effects and may influence the integrity of the cellular functions. The cost-effectiveness of cell-based therapies and harvesting of cells in sufficient quantities for translational applications are considerable limitations, unless the collection infrastructure is already in place, as is the case for RBC and platelets used in transfusion medicine [81]. In addition, restricted space of activity of circulatory-cells (e.g. RBCs) within blood vessels, short *ex vivo* shelf-life under standard conditions, and their potential of being damaged (osmotically/mechanically) during the loading of NPs or therapeutic molecules in these cells are further possible limitations to overcome. Hence, more research in this direction is needed in order to confirm the therapeutic benefits of these living cell carriers to brain targeting approaches.

9. Analyzing nanoformulations in complex biological matrices: common techniques and challenges

Nanocarriers behavior in biological fluids is vital for exploiting their beneficial properties. The physical and chemical properties of nanoformulation can change significantly, when they are exposed to complex biological matrices. These biological interactions could also pose potential risks or lower the efficacy of the therapeutic system. When nanoformulations are introduced into biological environments, several undesirable effects such as aggregation and non-specific absorption can occur due to changes in their surface properties. These surface properties of nanomaterials in a given medium are mainly governed by ionic strength, pH, temperature and the presence of biological macromolecules. As described earlier, protein corona is formed by plasma proteins as soon as NPs are introduced into the blood circulation and alters the biodistribution and clearance of NPs. In addition, the protein corona may hinder the specific interaction between NPs surface ligands and their receptor targets [292]. However, the influence of protein corona on brain-targeted drug delivery systems is evaluated by very limited studies. For example, incubating transferrin-conjugated NPs with serum-containing culture medium reduced the specific interaction between transferrin and transferrin receptor. This is because, proteins in the culture medium shielded transferrin from binding to its targeted receptors on cells and soluble transferrin receptors and the targeting specificity of transferrin-conjugated NPs was lost [244]. In another study, when 3.5 nm negatively charged SPIONs were incubated *in vitro* with 90% FBS, NPs adsorbed higher amounts of the small proteins ApoA-I and ApoA-II (≈30 and ≈11 kDa, respectively) compared to positively charged and plain 3.5 nm SPIONs. Once SPIONs were injected *in vivo*, ApoA-I and II were adsorbed on their surface and drove the NPs to brain vessels because these Apo(s) can cross the BBB through a saturable transport mechanism. This behavior may result in neurotoxic side-effects after injection of SPIONs [234]. In one earlier publication, ApoE was enriched on the surface of nanocarriers coated with the nonionic surfactant polysorbate 80 and hereby enabled the transport of nanocarriers across the BBB via receptor-mediated endocytosis [293].

To fully understand the *in vivo* fate of nanocarriers, it is important to understand how they behave in a complex biological environment, especially the physicochemical parameters of nanoformulations and the formation of protein corona. However, nanosurfaces and their interaction in the biological and cell surface media are difficult to characterize and to address this, appropriate analytical methods are needed. However, the small size, diverse composition and surface chemistry of NPs, and the highly complex nature of biological matrices make nanocarrier's detection and characterization challenging. Here, we have summarized different methods of nanoformulation

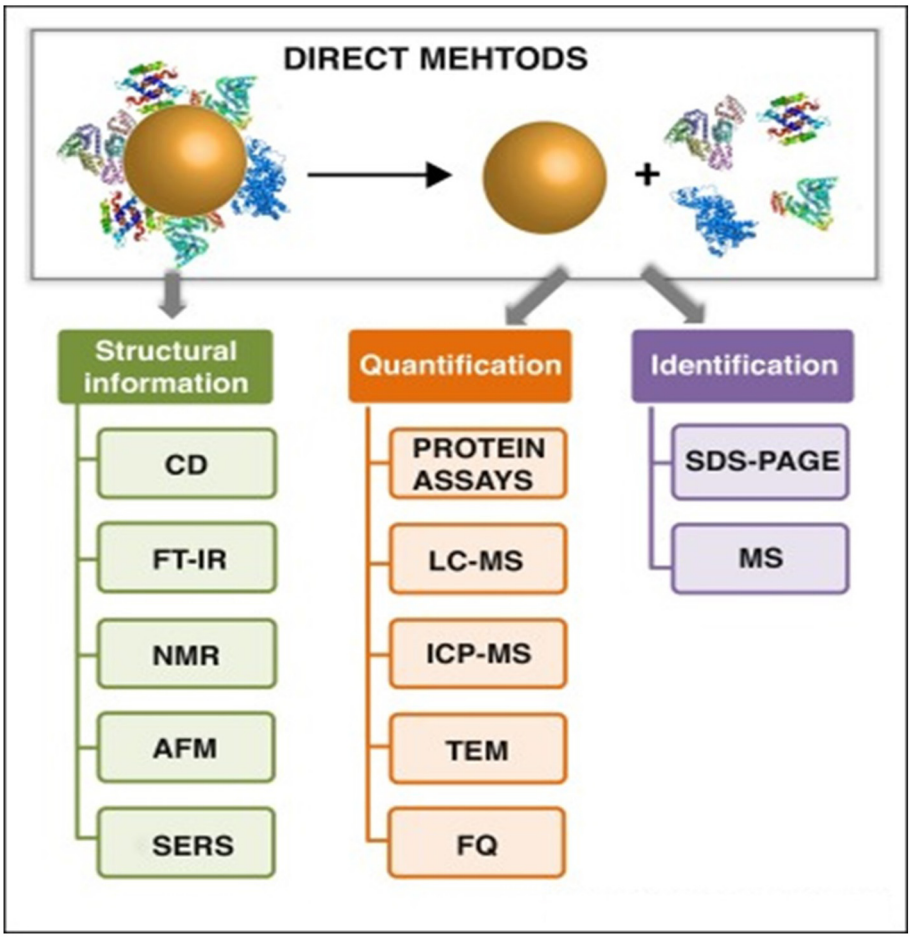

Fig. 7. A summary of the direct (Circular dichroism (CD), Fourier-transform infrared spectroscopy (FT-IR), Nuclear magnetic resonance (NMR), Atomic force microscopy (AFM), Surface-enhanced Raman scattering (SERS), Liquid chromatography–mass spectrometry (LC-MS), Transmission electron microscopy (TEM), SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis), etc.) analytical methods of protein corona evaluation. Reproduced with permission from [294].

characterization in terms of their advantages, limitations, analyzing parameters, etc., (Table 4). Since protein corona is of significant interest while discussing the NPs behavior *in vivo*, several techniques have been developed to evaluate the protein corona formation. The adsorbed proteins on NPs surfaces can be directly or indirectly analyzed as summarized in Fig. 7, and Fig. 8, respectively, (Figures reproduced with permission from [294]). Direct methods analyze the proteins that are adsorbed on the NP surface using spectrometry (e.g. MS, NMR, and FT-IR), circular dichroism, and microscopy methods (e.g. AFM, TEM), etc. In the indirect method, the protein corona is analyzed via measuring changes in the NP properties such as changes in size and aggregates, surface charge, density, mass, absorbance, and fluorescence. The measured parameters are then correlated with the amount of adsorbed proteins. In both cases, some techniques allow for detection of the protein corona *in situ*, while others require the removal of unbound proteins before the measurements.

Dynamic light scattering (DLS) is the most commonly used light scattering method to analyze NP agglomeration and size-distribution for monodispersed samples due to its ease of use and its applicability to a range of particle types and dispersion media. However, the results may be wrongly interpreted, since the scattering intensity may also be contributed by the components in the biological media. Moreover, selectively analyzing the relevant signals from NPs only is practically impossible. Gollwitzer *et al.* compared DLS, CLS (centrifugal liquid sedimentation), SAXS (small-angle X-ray scattering) and NTA (PTA: particle tracking analysis) techniques for the size measurement of silica NPs dispersed in water and in a cell culture medium [295]. The DLS results in the culture medium differed to a significant extent due to the presence of agglomerates, caused by the culture medium resulted in a significant increase in the size in PTA measurements, whereas the NP size was stable for SAXS and CLS measurements [295]. Recently, a depolarized DLS method has also been developed to circumvent the obstacles with polarized DLS and showed that unwanted scattering signals from proteins, not associated to the NPs, can be completely suppressed, thus resulting in an extraordinary signal-to-noise ratio [296]. A depiction of NPs and the bio-matrix background as seen in standard polarized and depolarized DLS experiments is represented in Fig. 9 (reproduced with permission from [296]). This approach offers opportunities addressing the behavior of NPs in complex biological environments.

Cryogenic plunge freezing and transmission electron microscopy (TEM) have been employed for the characterization of agglomeration states of NPs [297]. The approach relied upon freezing the NP dispersion at necessary speed to vitrify the liquid phase without significant reorganization of dispersed particles. Althoughsuitable for the size, shape, and composition analysis of individual particles, the method was not a reliable assessment of agglomeration, as the particulates present are known to aggregate together as the liquid phase recedes during evaporation [298]. Moreover, the data obtained from TEM are 2D in nature, which poses a limitation of employing this technique for agglomerates characterization. To this respect, 3D image reconstruction of cryogenic snapshot sampled agglomerates was recently used to calibrate the number of NPs per agglomerate, permitting precise description and comparison of the bioavailable doses in different biological environments [298].

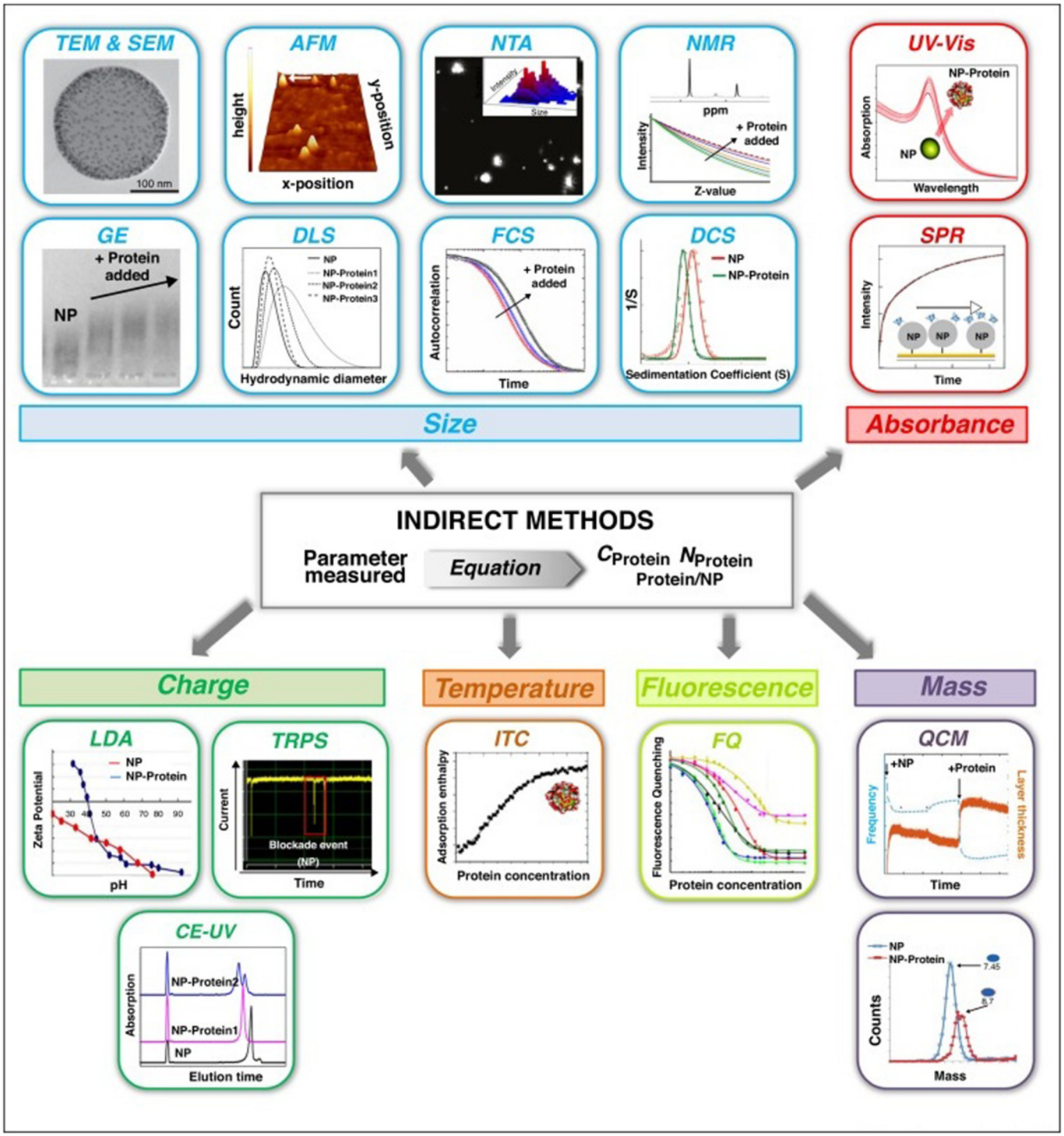

Fig. 8. A summary of the indirect analytical methods of protein corona evaluation. Reproduced with permission from [294].

The utility of tunable resistive pulse sensing (TRPS) technology has also been recently explored for the characterization of NPs dispersions in complex cell culture media [299]. The TRPS technology offered higher resolution and sensitivity compared to DLS and unique insights into nanomaterials size distribution and concentration, as well as particle behavior and morphology in complex media (Fig. 10, reproduced with permission from [299]). TRPS has been used for size and concentration analysis of particles, as well as to gather information on several other properties of NPs in solution. TRPS monitors changes in ionic current as individual NPs (or agglomerates) pass through an elastomeric membrane containing a single nanopore with a precisely controlled size. The particle-by-particle analysis by the TRPS offers great sensitivity and resolution for the characterization of NP dispersions in complex biological media but is currently limited to the counting of particles larger than ~50 nm. Minelli *et al.* used TRPS, differential centrifugal sedimentation (DCS) and DLS to measure the size of silica NPs in serum [300]. The DLS proved incapable of accurately measuring the NP size because of the presence of agglomerates whereas DCS and TRPS measurements were quite similar. In contrast to DLS and DCS, TRPS does particle-by-particle measurements, providing a statistical distribution of the results

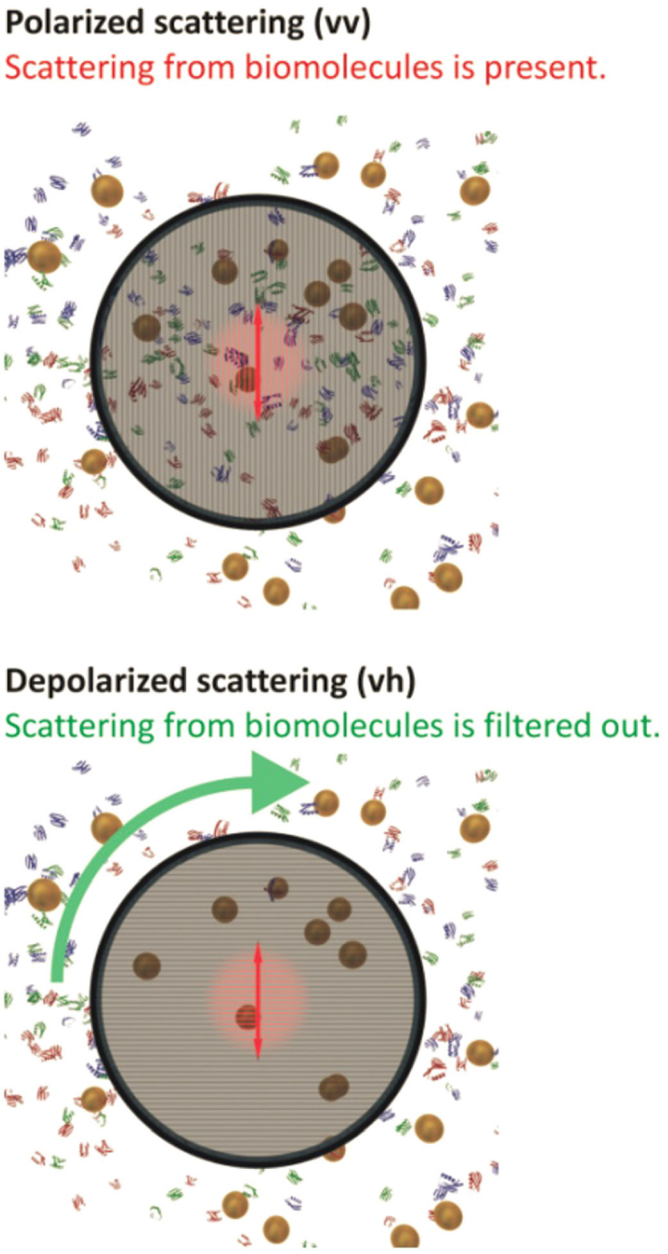

Fig. 9. Depiction of nanoparticles (NPs) and the bio-matrix background as seen in standard polarized (top) and depolarized (down) dynamic light scattering experiments, respectively. While in the first experiment scattering from the complex bio-matrix is clearly present, in the latter, depolarized scattering from the biological matrix is not visible, and thus, entirely negligible compared to the depolarized scattering of the nanoparticles. Owing to this, scattering exclusively from the NPs on an essentially zero-background is detected. Reproduced with permission from [296].

across an NP sample rather than average results. The study confirmed that TRPS is a sensitive and high-resolution technique in the characterization of NPs in biological media. Recently, microfluidic implementation of RPS (MRPS) has been made available that enables direct measurements of polydisperse biological NP samples such as protein aggregates or serum. By using microfluidic fabrication technology, the critical NP sensing constriction used in traditional RPS was shrunken by a factor of 100 or so, from a few tens of microns in diameter to a few hundred nanometers. This allows detecting much smaller particles using microfluidic RPS.

However, depending on the type of characterization technique applied, evident differences in the results may be generated. Hence, the use of complementary methods is crucial to get a better understanding of the NP physicochemical properties and protein corona formation. Using techniques that are complementary to each other is important in confirming the data and providing additional information on the same sample, including the fact that one technology can validate and complement the data from the second one. For example, the unique features of Nanoparticle Tracking Analysis (NTA) and DLS are two complementary techniques for the measurement of NP size and size distribution. The DLS measurement has a broad particle size range analysis capability, which is complemented by NTA's high-resolution single particle sizing capability. Another example is of using Atomic Force Microscopy (AFM) and Scanning Electron Microscopy (SEM) methods. These techniques resolve surface structure at the nanometer scale; however, the mechanisms of image formation are different, resulting in different information about the same surface structure. By using these two complimentary techniques, one technique may compensate for the imaging artifact of the other technique. One more example is of using SAXS method in analyzing the structural information of sample. SAXS provides average data from a large number of particles but does not provide the information about the shape or appearance of the sample. However, wide-angle X-ray scattering (WAXS) provides additional data on the composition, crystal structure, and size of the sample. Overall, using complementary techniques will often compensate for the limitations of the other technique in nanoformulations characterization. This is further justified by the fact that several of these analytical methods differ in suitability to certain particle size range, concentration, shape, user-friendliness, processing time, and in providing robust data, hence, the use of orthogonal complementary approach is quite necessary.

10. Challenges of implementing nanoformulations characterization methods in industrial settings and development

The physicochemical characteristics as well as the stability of nanomedicines under *in vitro* and *in vivo* environments are warranted to ensure the robust performance of the product. However, the lack of reliable and validated techniques to analyze nanomedicine characteristics under industrial Good Manufacturing Practice (cGMP) environment is challenging due to several reasons. Pharmaceutical industries are usually equipped with techniques best-suited for conventional formulations such as tablets, capsules, suspensions, etc., however, the characterization of nanomedicine products involves advanced and expensive techniques such as SEM, TEM, DLS, NTA, SAXS, NMR, etc. These techniques require a team of specialists to prepare the samples, conduct the analysis, and interpret the data. This further adds to the cost of nanoformulation characterization. In addition, the use of multiple analytical methods that complement each other to evaluate the same physicochemical property of nanoformulation is recommended, which is not easy in industrial settings. For example, DLS has size and shape constraints in the size-distribution analysis; hence, other methods such as NTA or electron microscopy (SEM/TEM) are needed in parallel. Any discrepancies in the results could alter the biodistribution, toxicity, or immunological profile of the nanocarriers. Therefore, using complementary techniques for characterizing NPs is highly advisable as explained in an earlier section.

Another important aspect is that the results can be influenced by the sample preparation methods, the medium used, and the way how samples are analyzed. Hence, it is also critical that the observations are real or the results affected by the factors involved in the nanoformulation measurements were understood correctly. Hence, there is a need to identify critical parameters in advance that can affect nanomaterial interaction with biological systems and for standardization of parameters, such as the sample preparation, testing conditions, etc. It is clear that developing robust characterization methods that are also compliant with GMP constraints is challenging. Identifying the appropriate methods to characterize the physicochemical or biological properties of nanomedicines is challenging from a technical as well as a regulatory

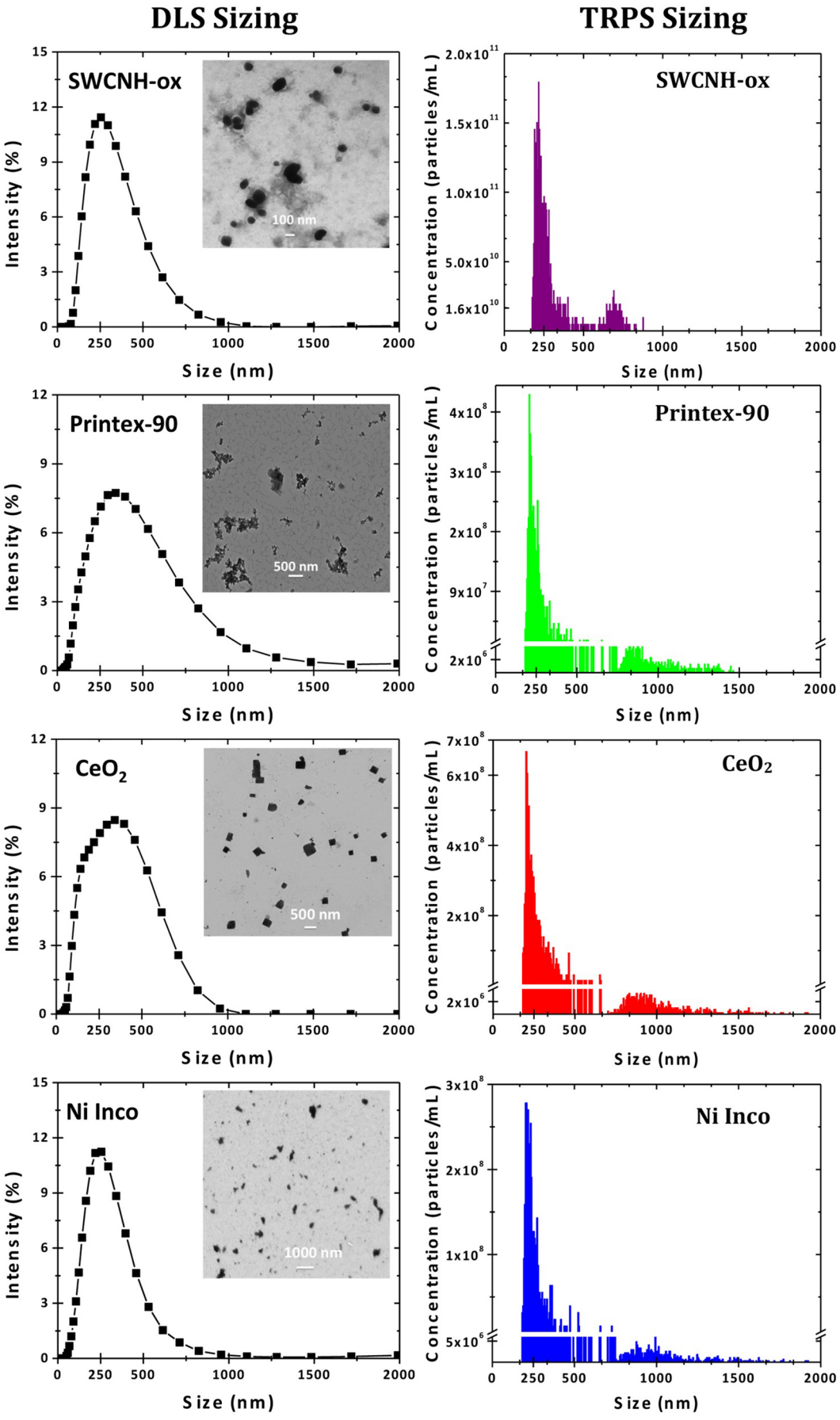

Fig. 10. Comparisons of engineered nanomaterials (ENM) size distributions as determined by dynamic light scattering (DLS, left panel), tunable resistive pulse sensing (TRPS, right panel), and transmission electron microscopy (TEM, inserts). A secondary peak related to agglomerates of primary particles c.a. 700-1000 nm is fully resolved in TRPS but absent in DLS. Note also that DLS size distributions are much broader than TRPS size distributions. Reproduced with permission from [299].

standpoint. In this context, regulatory relevance, reliability, accuracy, and validation of characterization methods to the extent they analyze correctly the biological effect are extremely important. Reproducibility and repeatability of intra- and inter-laboratory results is also vital and needs proper evaluation.

11. Conclusion

Nanoformulation systems have tremendous potential to solve the limitations of conventional therapies currently being used for various clinical indications, including for the treatment of neurodegenerative disorders. However, these novel systems have limitations in appropriately targeting the specific sites and have a poor capacity to overcome cellular barriers prior to releasing the encapsulated drugs. The biologically-inspired nanocarriers or cell-derived nanovesicles (e.g. RBCs, exosomes, and microvesicles) may avoid the above limitations of synthetic nanocarriers and are currently being exploited into several different applications as discussed in this manuscript. Although promising, the isolation, purification, characterization, and large-scale GMP production of cell-based systems are major challenges in their clinical translation and needs further evaluation. Nanoformulation biological fate is significantly affected by their physicochemical properties including size, shape, surface morphology, surface chemistry and functionality, hydrophobicity, elasticity, and many more. Hence, appropriate characterization of nanocarriers' *in vivo* and their interactions with biological systems is critical. In this regard, standardization of techniques and their robustness of providing accurate results in complex biological environments is of primary objective for drug delivery scientists.

During the development phases of nanocarriers, experiments should be designed to understand the impact of cell culture media, typically used to assess the efficacy, cellular uptake cytotoxicity, and therapeutic dose of nanocarriers on cells. The objective for these early experiments should allow a better understanding of designing nanomedicine products in the biological environment and for enhanced translational potential. Scientists need to design nanoformulation having in mind the specificities of the biological milieu(s) in which they are administered prior to reaching the targeted sites. However, the design complexity can be complicated as biological fluid may exhibit significant variability among patients and healthy individuals, and nanoformulation fate and properties will differ from patient-to-patient. Moreover, the composition of the protein corona of nanoformulation *in vivo* is influenced by the biological environment and the physical/chemical nature of the nanoformulation. Hence, the use of complementary characterization methods covering a wide range of parameters is crucial to get a better understanding of the nanocarriers' physicochemical properties and protein corona formation. Since the results can be influenced by the sample preparation method and the analytical protocols, it is also critical that the factors involved in the nanoformulation measurements are identified and optimized in advance. In this regard, to provide robust characterization for the identification of crucial parameters related to the effectiveness and safety of nanoformulations, organizations such as the Nanotechnology Characterization Laboratory (NCL) (https://ncl.cancer.gov/), National Institute of Standards and Technology (NIST) (https://www.nist.gov/), The US Food and Drug Administration (FDA) (https://www.fda.gov/), and European Nanomedicine Characterisation Laboratory (EU-NCL) (http://www.euncl.eu/) support the preclinical characterization of nanomedicines and provide a testing infrastructure covering a comprehensive set of assays to accelerate the product development and translation. The translational challenges and development strategies of nanomedicine formulations to make them a viable clinical product are recently reviewed [317].

There is now considerable research evidences in increasing the amount of drug delivered to the brain across the BBB using various formulation approaches as briefly discussed in the review. Yet, there are several issues hampering the clinical development of nanomedicine products for brain targeting; one is certainly the physicochemical complexity and characterization of nanoformulations in biological environment. This complicates the nanoformulations lab research and makes the large-scale development processes and scale up manufacturing difficult, especially when using the complex biological matrices. Hence, lab-scale development processes, characterization approaches, and targeting strategies of nanoformulations those with high translational potential needs to be designed/optimized early in the development processes. The translational challenges and development strategies of nanomedicine formulations to make them a viable clinical product are recently reviewed. In summary, the application of nanomedicines for drug delivery across the BBB has great potential, however, due to the complexity of the *in vivo* environment, limited understanding of the interactions between NPs and biological systems, rational designs and predictive modeling of the interactions between nanocarriers and biological systems using specialized and robust characterization methods are needed from early stages of the development in improving their therapeutic effectiveness, safety, and clinical translational potential.

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