Expert Opinion

Interactions of nanoparticles with plasma proteins: implication on clearance and toxicity of drug delivery systems

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Introduction: Intravenously injected nanoparticles, like any other foreign pathogen that enters the body, encounter multiple lines of defense intended to neutralize and eliminate the invading substance. Adsorption of plasma proteins on the nanoparticle surface is the first barrier of defense, which could lead to physical changes in the formulation, such as aggregation and charge neutralization, biochemical activation of defense cascades, and trigger elimination by multiple types of phagocytic cell.

Areas covered: In this review, recent knowledge on the mechanisms that govern the interactions of nanoparticles (micelles, liposomes, polymeric and inorganic nanoparticles) with plasma proteins is discussed. In particular, the role of the nanoparticle surface properties and protective polymer coating in these interactions is described. The mechanisms of protein adsorption on different nanoparticles are analyzed and the implications on the clearance, toxicity and efficacy of drug delivery are discussed. The review provides readers with the biological insight into the plasma/blood interactions of nanoparticles.

Expert opinion: The immune recognition of nanoparticles can seriously affect the drug delivery efficacy and toxicity. There is at present not enough knowledge on the mechanisms that dictate the nanoparticle immune recognition and stability in the biological milieu. Understanding the mechanisms of recognition will become an important part of nanoparticle design.

Keywords: clearance, nanoparticle, opsonization, toxicity

Expert Opin. Drug Deliv. (2011) 8(3):343-357

1. Introduction

Drug delivery systems are defined as supramolecular assemblies incorporating the cargo (drug, nucleic acid, toxin, etc.) intended to treat a disease. The carrier is intended to overcome the shortcomings of the cargo, such as unfavorable pharmacokinetics, poor solubility, instability, high toxicity, drug resistance and low cellular uptake. Since the seminal discovery of liposomes over 50 years ago by Bangham and Horne [1], there has been extensive research towards the development of new drug delivery systems. For some time, liposomes and emulsions dominated the drug delivery field. With the renewed interest in nanotechnology, new nano-sized formulations and nanomaterials have been developed [2]. These new materials include self-assembling polymers, protein nanoparticles, inorganic nanoparticles, fullerenes, carbon nanotubes, nanodiamonds and dendrimers. Owing to the urgent need to cure cancer, almost every developed nanoparticle is tested as a vehicle for delivery of chemotherapeutics to tumors. At the same time, most of these compounds are not likely to make it into the clinic owing to poor biocompatibility or potential toxicity. Thus, semiconductor quantum dots have been tested with success for simultaneous drug delivery and imaging of tumors [3], but the possibility of

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Article highlights.

- Protein interactions determine biodistribution, toxicity and efficacy of injected nanoparticles.
- Type of absorbed proteins depends on nanoparticle size, shape and surface properties.
- Liposomes and polymeric particles preferentially absorb lipoproteins, whereas inorganic and charged particles preferentially absorb blood contact factors.
- Apart from complement and immunoglobulin, the opsonizing effect of other nanoparticle-binding proteins is less clear.
- Nanoparticle elimination could be accomplished in opsonin-independent fashion through direct recognition by macrophages.
- Some proteins decrease interaction with macrophages and reduce toxicity.
- PEG coating prevents interactions with cells, blocks aggregation, but does not completely block protein absorption.
- Understanding of the mechanisms of immune recognition can promote fabrication of drug delivery systems with improved pharmacokinetics and efficacy.

This box summarizes key points contained in the article.

translation of these compounds in clinic is very unlikely owing to the apparent toxicity of the heavy metal components. On the opposite side, biodegradable systems such as polymers, micelles, protein-based and inorganic nanoparticles are already being tested in clinical trials or have been approved in patients; others are still at the stage of academic and industrial development [2].

With the accumulation of a large amount of data on the *in vivo* biodistribution and toxicity of the nanoparticulate systems, including human data, came a sobering realization that the living organisms possess very efficient barriers intended to block the invasion of foreign agents into the host territory, nanoparticulate drug delivery systems being no exception. The definition of 'foreign' is complex. It could be related to the surface presence of sugars, polysaccharides, proteins and nucleic acids in the combinations, conformations and in a context not known to the host. Also, the presence and spatial arrangement of certain chemical groups and atoms on the surface could be considered to be a foreign signal. Nanoparticles are highly organized clusters of chemical groups and/or biomolecules. Compared with classical (microscale) pathogens, nanoparticles can interact more efficiently with biological systems, owing to their higher surface area/volume ratio. On intravenous introduction of the nanoparticulate systems, interactions with the host occur, from the initial contact with blood/plasma to when the nanoparticles end up in the cells and tissues. These interactions could be divided into the following groups: i) immediate association with plasma proteins, cells and platelets; ii) time-dependent changes in the protein and cell association pattern during residence in blood; iii) activation of protein cascades; iv) recognition of the nanocarriers by the immune cells/macrophages; v) deposition of the nanocarriers in non-macrophage cells including tumor cells and healthy tissues; vi) intracellular processing and activation of signaling pathways/apoptosis.

Association of drug delivery systems with blood proteins is considered the most critical step, determining to a major extent the nanoparticle biodistribution, efficacy and toxicity. On binding of antibodies, complement and clotting factors, there is activation of contact defense responses, including clotting and complement cascade [4]. Interaction of nanoparticles with blood proteins can cause contact toxicity in the form of thrombosis and hypersensitivity [5-8]. Nanoparticle coating by plasma proteins can promote their macrophage uptake and elimination [7]. According to the fundamental rules of pharmacokinetics, the dose of nanomedicine that can reach the diseased tissue is directly proportional to the blood clearance [9]. Therefore, macrophage recognition can dramatically reduce the effectiveness of the nanoparticulate treatments. This problem is equally important for targeted nanomedicines that need time to bind to the tumor receptors [10], and for non-targeted nanoparticles that rely solely on the enhanced permeability retention (EPR) effect in the tumor [11]. In addition to the decreased efficiency, the uptake of drug-loaded nanoparticles (NPs) can result in damage to the immune cells due to both drug and nanomaterial [6,12,13]. Understanding the mechanisms of NP interaction with the body milieu could be instrumental in designing strategies to prevent the toxicity and premature clearance.

It is not possible to cover all the above aspects of nanoparticle immune recognition in one review. Therefore, this review focuses mostly on the current knowledge of interactions with blood proteins and the implications on toxicity and elimination by the reticuloendothelial system (RES). Other aspects of nanoparticle–protein interactions, such as physical studies of protein corona, complement activation and design of 'stealth' nanoparticles, are covered excellently in recent reviews by Aggarwa *et al.* [14], Lynch *et al.* [15], Moghimi *et al.* [7] and Szebeni [16].

2. Mechanisms of nanoparticle interactions with blood proteins

2.1 Methods used for studying plasma protein interactions with nanoparticles

Plasma proteome is an extremely complex system consisting of tens of thousands of proteins, with a dynamic range of > 13 orders of magnitude [17]. The highly abundant proteins include carrier/adaptor proteins secreted by solid tissues such as albumin and transferrin, and immunoglobulins. Intermediate and low-abundance proteins include receptor ligands and cytokines, tissue and cell leakage products, secretions and foreign proteins (**Table 1**).

According to the estimates, there are ~ 100,000 protein types and variants in plasma. This poses an enormous problem for characterization of proteins that adhere to nanoparticles during their contact with blood. A study of

Table 1. Abundance of different proteins in serum proteome.

Protein	Serum concentration	Abundance
Albumin	35 – 50 mg/ml	High
Transferrin	2 – 4 mg/ml	High
Fibrinogen	1.8 – 3.5 mg/ml	High
Alpha-2-macroglobulin	1 – 3.1 mg/ml	High
C5 complement	0.9 – 1.7 mg/ml	High
Apolipoprotein A-I	0.8 – 1/7 mg/ml	High
Haptoglobin	0.2 – 1.9 mg/ml	High
Ceruloplasmin	170 – 480 μg/ml	Moderate
Plasminogen	70 – 170 μg/ml	Moderate
IgD	< 80 μg/ml	Moderate
Retinol-binding protein	30 – 60 μg/ml	Moderate
Thyroxin-binding globulin (adult)	14 – 31 μg/ml	Moderate
Myoglobin	< 60 ng/ml	Low
Prostate-specific antigen	< 4 ng/ml	Low
Troponin	< 1.5 ng/ml	Low
TNF-α	< 20 pg/ml	Trace
Interleukin-10	15.6 pg/ml	Trace
Interleukin-β	–	Trace
Interleukin-5	–	Trace

protein adsorption to nanoparticles should include rigorous washing steps intended to remove thousands of unbound proteins from nanoparticles. Generally, this involves incubation of NPs with complex mixtures of proteins (e.g., plasma or serum) for various periods of time (often between 10 min and a few hours) and washing the unbound proteins using ultracentrifugation [18], column chromatography [4], or density gradient purification [19]. It can be safely assumed that three to four centrifugation washes can get rid of most of the plasma protein [18]. At the same time, there is a risk of overpurifying the sample and consequently losing weakly bound proteins. Cedervall *et al.* reported that loosely bound components of the protein corona detach from the nanoparticle surface during relatively mild washes [20]. Another report by Walczyk *et al.* on the protein corona of polystyrene nanoparticles suggests that the protein corona and the nanoparticle morphology are stable even after multiple centrifugation and washing steps [21]. Nevertheless, for nanoparticles with less stable protein association (e.g., hydrophilic polymer-coated ones) the processing can potentially introduce artefacts such as aggregation and changes in protein composition; necessary controls need to be carried out to account for these artefacts.

Following washing/enrichment steps, the proteins that adsorb on nanoparticles are eluted and analyzed. Usually, the proteins are separated with one- or two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis (2D-GE) is often used instead of one-dimensional gel electrophoresis [22]; some laboratories now apply this technique for gel-based proteomics analysis [23]. Antisera and specific antibodies are then used to identify the proteins [24]. Identification of the nanoparticle-binding proteins has been greatly facilitated by advances in proteomics and mass spectrometry. The protein band is excised from the gel, digested and identified by liquid chromatography-coupled mass spectrometry (LC-MS). In some nanoparticle-binding studies, 2D-GE with subsequent mass spectrometric analysis of the excised protein spots was used [25-27]. Two-dimensional gel electrophoresis is still a laborious and time-consuming technique with manual interventions. In addition, hydrophobic proteins, very large and very small proteins, and proteins that have extreme isoelectric point (pI) values are difficult to resolve with 2D-GE. Two-dimensional LC-MS, by contrast, is a truly shotgun approach in which many protein hits could be identified in one experiment. Especially attractive is a two-dimensional method with strong cation exchange (SCX) in one dimension and reverse-phase HPLC separation in a second dimension, before the use of an MS/MS instrument [28]. Such a technique was able to resolve thousands of plasma proteins over 10^4 dynamic concentration ranges [28], without the need for depletion of abundant proteins. Recently, a shotgun two-dimensional LC-MS/MS proteomics approach was used to analyze plasma proteins that bind to dextran-coated iron oxide nanoparticles [29].

In addition to the identification of adsorbed proteins, other parameters of nanoparticle–protein interactions, such as affinity and dissociation constants, surface charge and morphology, were studied using dynamic and static light scattering, surface plasmon resonance, gel electrophoresis, electron microscopy and circular dichroism. These studies have been informative with regard to the thermodynamics and kinetics of interaction and are reviewed elsewhere [30]. Thus, the interaction between plasma constituents and the nanoparticle surface is a complex dynamic process that involves thousands of competing species, which is dictated by protein concentration, affinity constant and on/off rate. Most of the kinetic studies conclude that the length of incubation and the protein/nanoparticle ratio are important determinants of the type and amount of absorbed protein [18,20,24]. The overall trend in protein adsorption is the change in total amount and the repertoire of adsorbed proteins over time [18,20,31]. Vroman *et al.* [32] first predicted this behavior of proteins adsorbing on artificial surfaces. Albumin and fibrinogen bind to polystyrene and poly-L-lactic (PLA) nanoparticles at earlier stages but are gradually displaced by proteins with higher affinity, such as apolipoproteins and immunoglobulins [20]. Incubation of lecithin-coated polystyrene nanoparticles with serum in a time-dependent manner showed a time-dependent increase in the coating of C3 complement and IgG, but not albumin, with the peak of absorption at 2 h [33]. Some of these studies involve simulated conditions with a few model proteins (BSA, fibrinogen), which might not reflect the actual situation in plasma [20,34,35]. Indeed, some of the proteins that bound to nanoparticles in the purified conditions did not bind when present in the context of the entire plasma proteome [36].

It should be kept in mind that most of the studies on the protein corona are performed using serum or plasma, and do not take into account the presence of blood cells, platelets and microparticles in the blood. All these can affect the interaction between nanoparticles and proteins in unpredictable ways.

2.2 Mechanisms of plasma protein adhesion to nanoparticles

Table 2 summarizes the identification of plasma proteins adhering to different nanoparticles using the methods described in the previous section. These data shed some light on the mechanisms by which proteins recognize nanoparticles. According to **Table 2**, the repertoire of proteins adhering to nanoparticles is relatively limited. For obvious reasons the surface chemistry plays the dominant role in the recognition, hence this parameter is discussed here in detail. Apolipoproteins are the main type of proteins coating liposomes and polymeric nanoparticles, but not inorganic nanoparticles. Apolipoprotein A-I (ApoA-I) is known to bind to the surfaces of liposomes and biomaterials [37]. The exchange of apolipoprotein between lipoproteins and nanoparticles that have hydrophobic domains was suggested to be the main mechanism of adsorption [31]. Using model polymer particles with decreasing hydrophobicity, Gessner *et al.* [38] demonstrated that ApoA-I, ApoA-IV, ApoC-III and ApoJ gradually disappear with decreasing hydrophobicity of the nanoparticle, whereas absorption of IgG and albumin does not change to the same extent. The most abundant proteins, albumin and fibrinogen, were found on many types of nanoparticle, but in view of the dynamic nature of the protein binding and the short incubation time for most of the experiments, it is not clear how relevant this absorption is to the *in vivo* situation. At the same time, cationic lipoplexes and polyplexes show strong albumin binding, probably because albumin is a negatively charged protein that has been shown to bind to cationic charges [39]. Albumin also shows affinity for hydrophobic surfaces and polyanions [40].

In contrast to polymer nanoparticles and nanoparticles with hydrophobic surface component, hydrophilic inorganic nanoparticles attract a different category of proteins, which could be defined as adaptor and carrier proteins, such as transferrin, haptoglobin, fetuin A (alpha-2-HS-glycoprotein), kininogen, histidine-rich glycoprotein, and contact (intrinsic) clotting pathway factors FXI and FXII. Most of these proteins are able to adhere to the anionic and metal surfaces, owing to the presence of positively charged domains (beta-2-glycoprotein) or histidine-rich sequences (histidine-rich glycoprotein).

Some of the proteins in **Table 2** specifically bind to defined chemical groups on the nanoparticle surface. For example, presence of hydroxyls (e.g., dextran and sugars) could promote the binding of C3 complement through its thioester group [41]. Mannose-binding lectins (MBLs) were shown to bind to sugar moieties of dextran-coated nanoparticles [29]. Kuroki *et al.* [42] demonstrated specific binding of serum mannose binding protein (MBP) to phosphatidylinositol (PI) liposomes. Dextran-coated particles appear to be recognized by antibodies, probably owing to the presence of sugar-binding antibodies against bacterial polysaccharides [43]. Ruoslahti and coworkers divided the nanoparticle-binding proteins into primary binders, that is, the ones that bind directly to the nanoparticle surface, and secondary ones, which are recruited through the primary binders. Thus, plasma prekallikrein and FXII apparently bind to foreign surfaces through kininogen, as these proteins circulate in a complex. In a similar fashion, MBL-associated serine proteases (MASP-1 and MASP-2) circulate in complexes with MBLs in plasma [44]. The same applies to complement assembly on the nanoparticle surface, where complement factors C5 – C9 and C1q are recruited following the IgG binding (classical pathway) or the C3 binding and activation (alternative pathway).

The effect of nanoparticle composition on the protein adsorption has been studied extensively. Nanoparticle surface hydrophobicity is an important determinant of the type and amount of bound protein. Several studies have demonstrated the role of nanoparticle hydrophobicity on the amount and profile of proteins adsorbed [18,38]. A common observation has been that hydrophobic particles bind more plasma proteins than their hydrophilic counterparts. In an earlier work by Moghimi and Patel, an important observation was made that liposomes rich in cholesterol bind less protein than cholesterol-free liposomes [45]. Liposomes composed of neutral saturated lipids with carbon chains greater than C16 have been reported to bind larger quantities of blood proteins compared with their C14 counterparts [4,24]. This has been explained by stronger affinities of plasma proteins, especially IgG and albumin for hydrophobic domains.

Nanoparticle surface charge is another important factor in protein interaction. Cullis and co-workers demonstrated that anionic liposomes including those containing phosphatidylserine (PS) bind more serum proteins than their neutral counterparts [4,24]. Phosphatidylserine liposomes have been shown to adsorb significantly more apolipoprotein E (ApoE) than their neutral counterparts [46]. Bradley *et al.* [47] reported binding of complement (C1q) to anionic liposomes. Significant plasma protein binding to vesicles containing cationic lipids (e.g., DMRIE) has been reported [48]. This may arise from electrostatic interactions between the cationic lipids and most of the negatively charged plasma proteins. Using negatively charged polymeric nanoparticles, Gessner *et al.* observed an increase in plasma protein adsorption with increasing surface charge density [23]. Other studies from the same group with polystyrene nanoparticles reveal that positively charged particles predominantly adsorb proteins with pI < 5.5, such as albumin, whereas negatively charged particles adsorb proteins with pI > 5.5, such as IgG [23,49].

Size and curvature of nanoparticles also appear to affect protein binding. For example, classical IgM-dependent complement activation is most efficient on dextran particles

Table 2. Identification of different components of nanoparticle protein corona.

Type of nanoparticle	Protein bound	Main mechanism of interaction	Ref.
100 nm dextran iron oxide (Feridex)	MBL-A, MBL-C, MASP, plasma kallikrein, high-molecular-mass kininogen, histidine-rich glycoprotein, phospholipid binding protein, beta-2-glycoprotein, alpha-1-antitrypsin, ApoB precursor, Factor FXII, FXI, fibronectin, vitronectin, complement C3	Charge	[19,29]
50 nm lecithin-coated polystyrene (LNS-50)	C3, IgG, ApoE, albumin	Hydrophobic, polar	[33]
Neutral liposomes (PC/PE/CH) with or without PEG 2000 5%	Fibrinogen, C3, fibronectin, albumin, IgG, alpha-2-macroglobulin	Hydrophobic, polar	[36]
Negative liposomes (70% PA) with or without + PEG 2000 5%	FXII, kallikrein, kininogen, fibrinogen C3, fibronectin, albumin, IgG, beta lipoprotein, alpha-2-macroglobulin, Factors B, H, V, Protein C, S, ApoA-I	Charge, hydrophobic	[36]
N-isopropylacrylamide (NIPAM) and *N*-tert-butylacrylamide (BAM) 50:50	ApoE, ApoA-I, -II, -IV, fibrinogen, ApoD, IgG, IgM, C4BP (complement), minor lipoprotein components	Polar	[18]
PC/CH/PS liposomes PC/CH/PG liposomes	Beta-2-glycoprotein, proportional to the percentage of negative lipid	Charge, hydrophobic	[66]
Single-walled carbon nanotubes TiO_2 (21 nm)	Alpha albumin Albumin, IgG, fibrinogen, ApoA-I, Complement C3, alpha-1-antitrypsin, fetuin A, alpha-2-acid glycoprotein, ApoD, clusterin	Non-ionic Charge	[83] [120]
SiO_2 (7 nm)	Albumin, IgG, fibrinogen, ApoA-I, C3, alpha-1-antitrypsin, alpha-2-acid glycoprotein, ApoD, clusterin	Charge	[120]
ZnO (30 nm)	Albumin, IgG, fibrinogen, ApoA-I, C3, transferrin, haptoglobin	Charge	[120]
PLA; PEG-PCL, PEG-PLA 160 – 270 nm	Fibrinogen (α, β, γ chains), IgG, ApoA-IV, ApoE, ApoA-I, ApoC-III	Polar	[27]
Isobutylcyanoacrylate Dextran coated	ApoA-I, ApoC-II, fibrinogen	Polar	[121]
Gold colloidal 50 nm	Fibrinogen, inter alpha-trypsin, gelsolin, kininogen, alpha-1-antitrypsin, protocadherin, Crumbs protein, tropomyosin, alpha-actin, C3, vitronectin, IgG, microcephalin, albumin	Charge	[25]
Gold colloidal 30 nm	Fibrinogen, inter alpha-trypsin, gelsolin, kininogen, a-antitrypsin, protocadherin, Crumbs protein, tropomyosin, alpha-actin, C3, C4, vitronectin, IgG, microcephalin, albumin, C1q, ApoD, ApoA1, ApoE	Charge	[25]
PEG-hexadecyl cyanoacrylate, 135 nm	ApoAI, AII, AIV, CII, CIII, ApoE, ApoJ, a-antitrypsin, alpha 2-HS glycoprotein, transthyretin, IgG, IgG, IgM	Polar, hydrophobic	[26]

Table 2. Identification of different components of nanoparticle protein corona (continued).

Type of nanoparticle	Protein bound	Main mechanism of interaction	Ref.
Cetyl palmitate/poloxamer 407	ApoA-I, II, IV, ApoC-II, C-III, ApoJ, transthyretin, IgG, IgM, alpha-1-antitrypsin, haptoglobin	Hydrophobic	[31]
Latex	Albumin, fibrinogen, ApoA-I, ApoA-IV, ApoC-II, ApoJ, a-antitrypsin, IgG, IgM, IgD	Hydrophobic	[38]
Liposomes PC/CH/CL (35:45:10) PC/CH/DOPA) PC/CH/DOPS	C3/C3b, IgG	Hydrophobic, polar	[24]
Liposomes SM/PC/G_{M1} SM/PG/G_{M1}	No C3/C3b, IgG		
Liposomes DSPC:CH:DSPE-PEG2000 (50:45:5) DSPC:DSPE-PEG2000 (99:1 and 95:5)	Albumin, IgG	Hydrophobic, polar	[94]
Liposomes PI (100%) PS (100%) PG (100%) PC (100%)	Mannose binding protein (MBP)	Hydrophobic, charge	[42]
Liposomes PC: CH: DOPS (35:45:20) PC: CH: DOPA (35:45:20) PC: CH: CL (35:45:10)	β2-Glycoprotein I	Hydrophobic, charge	[64]
DiC14 amidine liposome: plasmid DNA complex	Apolipoprotein (C-I, C-II, C-III, A-1, A-II), alpha-1-antitrypsin, fibrinogen (α, β, γ chains), haptoglobin, serotransferrin, transthyretin, IgG	Hydrophobic, charge	[72]
Liposomes PC (100%)	ApoA-I, ApoA-II, ApoA-IV, ApoC and ApoE	Hydrophobic	[62]
Liposomes Egg PC (100%)	ApoE ApoA-I ApoA-IV	Hydrophobic	[61]
Nanospheres (polymer: PLGA, PLA, PCL)	IgG, C3/C3b	Polar	[122]

in the optimal size range, ~ 250 nm [50], whereas larger particles do not attract as much IgM and therefore do not activate to the same extent. The same phenomenon of size-dependent activation of complement was observed for liposomes [51]. Dobrovolskaia and co-workers reported that significantly more protein was adsorbed on 30 nm than on 50 nm gold particles (**Table 2**) [25]. Lynch *et al.* [15] studied the role of particle size and surface area on the protein adsorption by NIPAM/BAM (50:50) copolymer nanoparticles. Using nanoparticles varying in size between 70 and 700 nm, they demonstrated that the amount of bound plasma proteins increased with increasing available surface area at a constant particle weight. At a constant weight fraction of nanoparticles, the surface area available for protein binding increases with decreasing particle size. Another study involving the interaction of gold nanoparticles with common plasma proteins suggests that the thickness of the adsorbed protein layer increases progressively with nanoparticle size [34].

2.3 Role of plasma proteins in clearance by macrophages

There have been numerous studies dedicated to understanding the role of plasma proteins in the uptake of nanoparticles by macrophages. The current concept is that the presence of protein corona should have an impact on the nanoparticle's presentation to the immune cells and macrophages [30]. With most bound proteins having been identified and rounded up, uncertainty still exists whether these proteins promote the uptake of nanoparticles *in vitro* and *in vivo*. Some plasma IgG and complement components C1q and C3b (iC3b) are known to trigger phagocytosis of viruses and bacteria by macrophages through recognition by Fc-gamma and complement receptors CR1 and CR3, respectively [52]. In the experiments *in vitro*, coating liposomes with immunoglobulins greatly increases their uptake by primary macrophages [53]. Positive correlation between complement C3 adsorption and uptake/clearance has been observed for liposomes, polylactic acid and poly(methyl methacrylate) nanoparticles particles [24,54-56]. On the other hand, the role of other proteins in the macrophage uptake is less clear. For example, fibronectin is mainly devoid of opsonizing activity for many types of pathogen [57,58], but it has been shown to promote uptake of silica nanoparticles by cultured macrophages [59] and of liposomes by peritoneal macrophages [53]. Tumor cell uptake of iron oxide nanoparticles in serum has been shown by Moore *et al.* [19] to increase twofold over control particles. They indentified fibronectin, vitronectin and complement C3 as the nanoparticle-bound proteins.

ApoB and ApoE have been shown to play a role in the uptake of liposomes by hepatocytes [60,61]. An interesting mechanism of uptake of egg PC liposomes by hepatocytes was suggested [62]: adsorption of ApoE-directed liposomes to hepatocytes, whereas other apolipoproteins negated this effect by displacing ApoE. These results are in contrast to poly-L-lactic nanoparticles, where apolipoproteins did not affect the uptake [55].

The main problem of *in vitro* experiments is that the macrophages are taken out of their tissue context. To solve this problem, several groups performed *ex vivo* perfusion studies of rat liver. In such studies, the role of individual components of serum could be analyzed. Thus, liver perfusion studies with HEPC/CH/DCP liposomes in the absence or in the presence of serum showed that C3 complement plays a major role in the uptake of these liposomes [63]. Several authors attempted to dissect the role of plasma proteins by using knockout mouse models deficient in nanoparticle-binding proteins. Simberg *et al.* analyzed the biodistribution of iron oxide nanoparticles in mice deficient in complement C3, kininogen, histidine-rich glycoprotein, fibronectin, fibrinogen and mannose-binding lectin [29]. The results were mostly negative, that is, the deficiency of plasma opsonins, including C3 and immunoglobulin, had no effect on the clearance. Chonn *et al.* [64] found a correlation between the absorption of beta-2-glycoprotein onto negatively charged liposomes and their liver clearance. However, Yan and co-workers [65,66] tested the effect of beta-2-glycoprotein and ApoE on the macrophage uptake of negatively charged phosphatidylserine and phosphatidylglycerol liposomes (beta-2-glycoprotein is known to adsorb onto negatively charged surfaces [67]) using knockout mice; the conclusion was that these proteins do not affect the biodistribution to the liver macrophages and hepatocytes. The lack of effect of individual knockouts could be explained by the redundancy of the macrophage uptake system.

Several authors attempted to identify proteins after isolating nanoparticles following *in vivo* injection, or by measuring depletion of plasma protein following injection, and to correlate with the clearance. Chonn *et al.* [24] found that GM1 liposomes adsorb less protein such as IgG and C3 than other types of liposome following intravenous injection into a mouse. They found a direct correlation between the amount of adsorbed protein and the clearance times. Schreier *et al.* [68] observed that plasma fibronectin is temporarily depleted following injection of large (0.5 μm) liposomes, and suggested the involvement of fibronectin in liposomal clearance. One of the most important reports on clinically relevant interaction between liposomal drug delivery vehicle and plasma protein in patients has been published recently. In this study, amphotericin B liposomes were injected into patients and the association of liposomes with lipoprotein fractions was determined [69]. The liposomes were found to be associated with high-density lipoprotein fraction in plasma. This strategy of isolating the 'bait' (protein) rather than the 'prey' (nanoparticle) gave interesting information on the distribution of nanoparticles among different plasma protein fractions, and also suggests that the biodistribution of liposomes *in vivo* could be affected by lipoprotein binding.

As opposed to the opsonizing action of plasma proteins, in some cases, by masking the nanoparticle charge and surface, the proteins could work as dysopsonins [70]. Pre-coating poly(methyl methacrylate) (PMMA) nanoparticles with plasma has been shown to decrease their liver uptake *in vivo*,

suggesting the involvement of dysopsonins [56]. The inhibitory effect of serum on the hepatic uptake of liposomes is species-specific [63]. Anionic liposomes coated with plasma or β_2-glycoprotein I showed decreased uptake by liver cells *in vitro* [66]. Human serum albumin (HSA), when adsorbed on the surface of polystyrene microparticles, was reported to inhibit their phagocytosis by dendritic cells [71]. Absorption of apolipoproteins negatively correlated with transfection efficacy of DiC14 amidine liposome lipoplexes *in vitro* [72].

The fact that serum and plasma proteins often interfere, rather than promote nanoparticle and liposome uptake, suggests that some of the uptake could be mediated by means of direct recognition of the nanoparticle surface. Many types of nanoparticle and pathogen, including gold, silica and liposomes, can be taken up by macrophages in the absence of serum [55,73,74]. This is more evidence that macrophages are equipped with an effective system to recognize nanoparticles even in the absence of opsonization signals. This hypothesis is difficult to test *in vivo* in view of the constant presence of plasma proteins. Several studies using *ex vivo* perfusion have shown that the liver uptake of lecithin polystyrene nanoparticles [75], PMMA nanoparticles [56] and liposomes [63] is reduced by preincubation with serum. A broad group of macrophage receptors could be responsible for opsonin-independent clearance of different nanoparticles. Nanoparticles carry repetitive chemical patterns such as ligands and chemical groups on the surface. Pattern recognition receptors, such as Toll-like receptors and C-type lectins, are able to recognize bacterial and fungal polysaccharides, although some of these receptors lack professional phagocytic function [76,77] and trigger endocytosis indirectly. Macrophage scavenger receptors (SRs) are a broad group of phagocytic receptors that are responsible for the elimination of blood-borne viruses, pathogens and negatively charged ligands [78]. Some of these receptors (SR-As) are essentially restricted in expression to the macrophages; others, such as receptor CD36, are expressed on several cell types, including macrophages, platelets and endothelia. Several new macrophage scavenger receptors for phosphatidylserine have been found recently [79,80], although these receptors are not expressed in the liver. A typical scavenger receptors class A-I/II recognizes negatively charged surfaces through positively charged collagen-like domain (CLD) [81], and it is likely that this domain participates in the recognition of negatively charged nanoparticles. Several reports have shown that polyanionic ligands of scavenger receptors, including polyinosinic acid, fucoidan and dextran sulfate, could inhibit the uptake of quantum dots [82], carbon nanotubes [83], iron oxide [84] and polystyrene [85].

2.4 Effect of polymer protective coating on the nanoparticle protein coating and clearance

Decorating the surface of nanoparticles with repelling hydrophilic polymers improves circulation properties and decreases macrophage recognition of many types of nanoparticle (reviewed by Moghimi *et al.* [7]). The best example is long-chain polyethylene glycol (PEG), although other hydrophilic polymers such as Pluronic F68 [86-88] and Poloxamer (block copolymer of polyethylene oxide and polypropylene oxide [89]) have been used. The surface grafting strategy with PEG has been widely used with many types of nanoparticle and is being used in commercial liposomal doxorubicin formulation Doxil® Centrocor Ortho Biotech (Horsham, PA). Another example of stealth coating is crosslinked hydrogel, including polyvinylpyrrolidone [90] and crosslinked dextran iron oxide nanoparticles [91]. The preparation of the latter is based on reacting 1-chloro-2,3-epoxypropane (epichlorohydrin) with dextran-coated nanoparticles in basic conditions with subsequent formation of a steric layer of crosslinked hydrogel.

Despite the widespread use of polymer coatings for prolonging the circulation of nanoparticles, the mechanism of action is not entirely clear. Torchilin and Trubetskoi [92] discussed the mechanism of action of polymers in imparting long-circulating properties. According to their model, long chains of polymers form a random cloud around the nanoparticle, thereby preventing protein absorption. Indeed, PEG has been shown to create a brush border around nanoparticles and provide a nonspecific impermeable barrier that sterically prevents access of proteins [93]. Length and density of polymer would be critical in obscuring the nanoparticle surface from the interactions [9,93]. This model explains only partially the stealth effect of crosslinked hydrogels, where the conformational freedom and flexibility is much more limited than that of PEG chains. In addition, for some nanoparticle types, there is no direct correlation between the amount of protein coating and macrophage uptake. Indeed, SDS–PAGE analysis showed that there is little or no effect of protective PEG coating on the type or the amount of plasma proteins absorbed on liposomes and hydrogel-coated inorganic nanoparticles [36,94,95]. For PEG-hexadecyl cyanoacrylate nanoparticles (135 nm), PEG was able to reduce the adsorption of ApoC and immunoglobulins, but not of other proteins [26]. In the case of PLGA nanoparticles, there was a significant reduction in the protein adsorption as verified by 2D-GE, but even with 5% PEG 5000 there was substantial protein binding [27]. At the same time, for neutral liposomes composed of phosphatidylcholine and cholesterol, and for negatively charged liposomes composed of phosphatidic acid, no effect of PEG-DSPE 2000 addition on the protein adsorption was found [36]. Price *et al.* [36] reported an inverse relationship between PEG coating of negatively charged phosphatidic acid liposomes and purified fibrinogen adsorption, but in the same work the PEGylation had no effect on total protein adsorbed from plasma. These studies demonstrate that the role of protective stealth coating might be more complex than simply prevention of protein absorption, and other explanations, such as direct inhibition of nanoparticle binding to macrophage surface [46], are worth considering. It is likely that because each nanoparticle type attracts a different set of proteins, the protective mechanism of PEG could be different for each type of nanoparticle.

Besides the prevention of plasma protein binding, other mechanisms of stealth effect of polymers are possible. Thus, DSPC liposomes without PEG show a higher level of aggregation than with PEG [94], which could affect spleen filtration (see below). PEG coating has a strong effect on colloidal stability by preventing aggregation in the presence of salt [96]. Dos Santos *et al.* suggested that the colloidal stability imparted by PEGylated layer could be the explanation of long-circulating properties of nanoparticles [94]. It is also likely that prevention of aggregation and decreased receptor-mediated recognition are both important for PEG stealth properties.

Despite significant prolongation of nanoparticle clearance by PEG and hydrogel coating, liver and spleen macrophage accumulation is still an issue, with > 50% of the injected dose ending up in these organs after 48 h circulation [9,93,97,98]. Recently, several reports demonstrated the accelerated clearance of PEGylated liposomes from circulation [99]. The effect was attributed to spleen-dependent generation of specific IgM antibodies that trigger complement absorption [99]. Some curious phenomena were observed. Preinjection with plain liposomes triggered the clearance of a second injection of PEGylated liposomes, suggesting immunogenicity of lipids as one of the factors. Also, the effect disappeared with time. It is not clear, however, whether this effect is relevant to humans.

Hydrogel-coated crosslinked iron oxide nanoparticles show prolonged circulation times and delayed macrophage clearance *in vivo*. However, even though hydrogel nanoparticles manage to escape premature recognition by the liver and spleen, they eventually end up in the RES organs (**Figure 1**). Dextran is known to activate complement through C3-dependent and lectin-dependent mechanisms [41]. Some researchers attributed activation of complement to the presence of hydroxyl groups [100]. It is possible that hydroxyl groups trigger the clearance of crosslinked dextran by means of complement activation.

Another problem of nanoparticle stealth coatings is directly related to the surface-repelling action of the brush layer. Thus, there is an apparent decrease of affinity of surface-tethered ligands owing to the interference of the surrounding PEG brush layer [101]. Peptide-mediated targeting should be especially sensitive to the presence of PEG owing to the modest affinity and smaller size. Inclusion of PEG above 1% in the cationic lipoplexes has been shown to reduce greatly the efficacy of siRNA delivery to organs and cells, owing to the reduced ability of cationic nanoparticles to interact with the negatively charged cell surface [102].

2.5 Effect of plasma proteins on toxicity and efficacy

Many clinically approved drug delivery systems show some degree of toxicity related to the triggered immune reactions and complement activation [16]. Signs of complement activation and anaphylactic C5a generation, such as hypotension and anaphylaxis, facial flushing, dyspnea, tachypnea, facial swelling, headache, chills, hypotension or hypertension, chest pain and back pain, have been observed with many clinically used nanoparticles. Szebeni [16] and Andersen *et al.* [103] reviewed the mechanisms and clinical cases of complement activation by drug delivery vehicles. Taxol™, Bristol-Myers Squibb, New York, NY (paclitaxel emulsion in Cremophor EL) [104], liposomal doxorubicin (Doxil [105]) and liposomal daunorubicin (DaunoXome™, Gilead Sciences, San Dimos, CA [106]) have been shown to have adverse reactions after intravenous injection in a significant proportion of patients. Dextran iron oxide (ferumoxides, Feridex™) demonstrated similar complement-dependent side effects on intravenous administration, which led to withdrawal of the product from the market by the manufacturer. Again, most of these effects could be attributed to complement activation by lipid components of the formulation, or by dextran coating of iron oxide in the case of Feridex.

Intravenously injected nanoparticles can induce platelet activation and microthrombosis in peripheral vasculature. Negatively and positively charged particles, such as amine and carboxyl quantum dots, have been shown to induce platelet aggregation *in vitro* and intravascular thrombosis in lungs [51]. Platelet aggregation and activation has also been shown for carbon nanotubes and for negatively and positively charged polystyrene nanoparticles [5], but it is not clear whether these nanoparticles induce thrombosis *in vivo*. Many types of inorganic nanomaterial, including quantum dots, have been shown to induce microthrombi in vital organs [51], and a targeted approach to induce tumor infarction by thrombogenic iron oxide nanoparticles has been reported [107]. On the other hand, Dobrovolskaia and co-workers [25] performed an extensive study on the blood contact properties of colloidal gold nanoparticles. The conclusion was that for these particles no platelet activation and thrombosis (thrombin generation) was induced. Therefore, the conclusion is that the thrombogenicity of nanoparticles is surface dependent. Several mechanisms can explain surface-triggered thrombosis. The intrinsic (contact) clotting pathway starts with absorption of Factor FXII, Factor FXI, prekallikrein and kininogen on the negatively charged surface. Many types of metal nanoparticle and liposome adsorb these factors (**Table 2**), but the role of the intrinsic clotting pathway in nanoparticle-induced thrombosis has not been conclusively proven [108]. On the other hand, many types of foreign surface promote the thrombosis by activation of tissue factor (FIII) expression by monocytes [108].

Another undesirable consequence of nanoparticle–protein interaction is aggregation. Aggregation can be mediated through several mechanisms, including surface charge neutralization by proteins and polyvalent anions and cations, lateral lipid separation, non-bilayer transitions, exposure of hydrophobic domains, fusion and bridging of individual particles by proteins. Aggregation is a surface-dependent phenomenon. Usually, strongly cationic and strongly anionic particles induce a higher level of aggregation. Thus, using differential centrifugation method, Walczyk *et al.* [21] demonstrated that

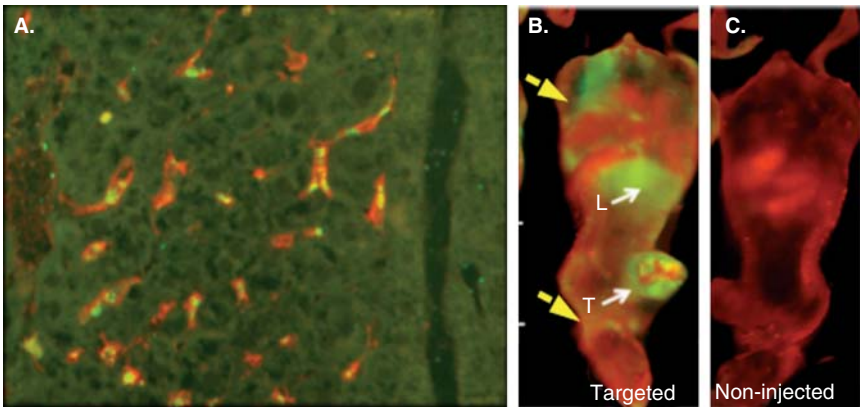

Figure 1. Biodistribution of long-circulating hydrogel-coated crosslinked iron oxide in mice. Nanoparticles were injected intravenously. **A.** Liver tissue histological staining. CLIO (green) colocalizes with Kupffer cell staining (red) 24 h post-injection. **B.** Whole-animal imaging (using LI-COR Odyssey instrument) of $\alpha_V\beta_3$ integrin-targeted, Cy7-labeled CLIO 24 h post-injection. Arrows show areas of accumulation of nanoparticles (green, 720 nm emission) in the tumor, liver, spleen and lymph nodes. **C.** Non-injected mouse control. Red is the body autofluorescence at 670 nm emission wavelength.
Colour is available online at www.informahealthcare/edd.
CLIO: Crosslinked iron oxide.

sulfated polystyrene particles are much more prone to aggregation than carboxylated ones. The reason for this is not clear, but could be ascribed to the efficiency of charge neutralization by the protein corona and to the bridging between individual nanoparticles by plasma proteins. Using dynamic light scattering, Rausch *et al.* [109] demonstrated aggregation of poly-L-lysine nanoparticles in serum; surface PEGylation above 8% prevented the aggregation process.

The main untoward consequence of aggregation is the accelerated clearance from circulation. Splenic filtration of poloxamine-908-coated polystyrene spheres of 0.2 µm diameter by red pulp was demonstrated [110]. The filtration could be responsible for the elimination of circulating aggregates, although this effect is likely to be species dependent. Liver macrophages also show higher uptake of larger particles [111].

Liposomal and micellar systems, as opposed to polymeric and inorganic nanoparticles, appear to be particularly labile in the presence of serum. Dye-loaded phosphatidylcholine liposomes showed release of the dye on incubation with serum [62]. Copolymer micelles of polyethylene glycol-*b*-poly(5-benzyloxy-trimethylene carbonate) loaded with ellipticine, a hydrophobic anticancer drug, showed ~ 40% release of the drug from micelles into the protein phase [112]. The reason for this instability could be the affinity of plasma proteins such as lipoproteins for hydrophobic domains of ampiphilic assemblies. On the other hand, cationic lipoplexes used for nucleic acid delivery and transfection are prone to the degradation in serum owing to strong interactions between the cationic bilayer and anionic plasma components and cells, with subsequent aggregation, disintegration and potential destruction of the nucleic acid payload [113,114]. The inhibitory effect of serum on lipoplex and polyplex transfection efficiency is a well-recognized phenomenon. Degradation of DOTAP/DOPE liposomes in serum was demonstrated with electron microscopy [113] and by fluorescence methods [115]. PEG coating of lipoplexes [116] and inclusion of strong polycations in the lipoplex formulation [117] could negate some of the deleterious effects of plasma, such as DNA-lipid dissociation and rapid clearance.

In some instances, interaction of cationic nanoparticles with plasma proteins can actually decrease toxicity. Thus, cationic polyamidoamine dendrimers have been shown to interact in PBS with red blood cell membranes and to have hemolytic action [118]. It was suggested that strong interactions of these nanoparticles with serum albumin [35] could prevent some of the toxicity when dendrimers come into contact with blood [118]. Cationic charge neutralization by anionic groups of albumin was suggested as the main reason for the protective effect [119].

3. Conclusions and future outlook

Nanoparticulate drug delivery systems are considered foreign by the body. Multiple defense mechanisms with overlapping specificities recognize and sequester extremely efficiently the injected foreign materials. According to **Figure 2**, the recognition could be opsonin-dependent, opsonin-independent, or a combination of both. The degree of 'foreignness' will determine whether the nanoparticle will undergo clearance by the RES system and activate defense pathways with resulting toxicity. The interactions with plasma proteins and plasma factors affect clearance, systemic toxicity and efficacy of nano-sized drug delivery vehicles. Nanoparticle surface properties, coating, size and shape all affect the protein interaction. There is a complex relationship between the nanoparticle composition, nature of absorbed proteins and the physiological (pathological) effect. For certain types of nanoparticle, the adsorbed proteins could affect the toxicity, aggregation and clearance, for others there could be a reduction

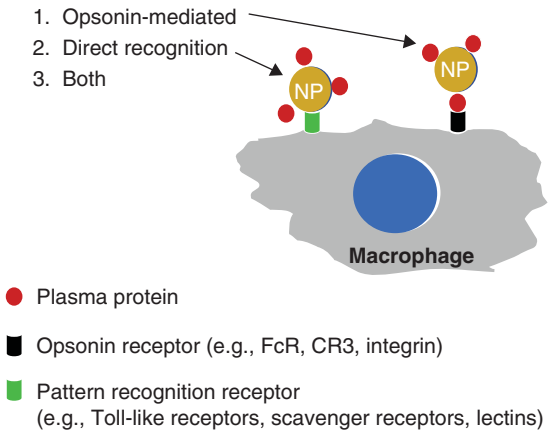

Figure 2. Scheme of possible mechanisms of nanoparticle recognition by macrophages. Following intravenous injection, plasma proteins coat nanoparticles. Macrophages can recognize NPs either directly or through the protein corona.
NP: Nanoparticle.

in these effects following protein absorption. As the premature recognition and elimination of nanoscale drug/gene delivery systems is one of the most serious limitations of *in vivo* nanomedicine, better understanding of the parameters that affect the interaction could improve the design of 'stealth' nanoparticle coating for more efficient and safe drug delivery.

4. Expert opinion

There is not enough knowledge at present on the mechanisms that dictate the nanoparticle immune recognition and stability in the biological milieu. It appears that there is no general rule that could be applied to every type of nanomaterial to predict the outcome; the rules applicable for one type of nanoparticle might not be relevant to another. Understanding better the role of nanoparticles' physicochemical parameters in protein and receptor recognition could be a game-changer in the design of drug delivery vehicles, akin to the drug design that revolutionized the drug industry and modern medicine. How could this knowledge be used? For example, nanoparticle coating could be designed to repel better the interactions with relevant receptors and proteins. Combining engineering, chemistry and computation modeling of the protein interactions could become an important part in the design of drug delivery systems in the future.

In pursuing the issue of prolonging circulation time of drug delivery systems, one should ask the question 'How long a circulation time is needed?' Liver and spleen perform the important function of filtering out toxic compounds, so a balance between RES elimination and residence time in blood is important. Drug delivery systems that circulate for prolonged times might end up in healthy tissues, even when targeting ligand is present, as the normal cells are also capable of uptake. The ideal drug delivery system should have minimal immune activation and contact toxicity, but at the same time avoid pollution of healthy organs and tissues, which would negate the purpose of a drug delivery vehicle. A strategy to trigger the accelerated systemic clearance of injected nanoparticles after a sufficient amount of drug has accumulated in the target organ is one potential solution to this problem.

Declaration of interest

The authors state no onflict of interest and have received no payment in preparation of this manuscript.

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