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Keynote Review

Overcoming the protein corona in chitosan-based nanoparticles

Anna E. Caprifico[1], Peter J. S. Foot[1], Elena Polycarpou[1] and Gianpiero Calabrese[1]

[1]School of Life Sciences, Pharmacy and Chemistry, Kingston University London, Penrhyn Road, Kingston upon Thames, United Kingdom, KT1 2EE

Corresponding author: Calabrese, G. (G.Calabrese@kingston.ac.uk) tel: +44 (0) 20 8417 7065

Abstract

Numerous properties of chitosan have led to its extensive use in the formulation of nanomaterials for drug delivery. However, the cationic surface of chitosan-based nanoparticles adsorbs proteins upon exposure to biological fluids, forming a phenomenon known as “protein corona”. This causes several effects such as decreased bioavailability and limited in vivo clinical applications of chitosan nanoparticles. Understanding and overcoming the effects of protein adsorption on chitosan nanoparticles is key for drug delivery purposes. This review focuses on the strategies implemented to increase the stability of chitosan nanoparticles in the systemic circulation by averting the formation of protein corona and the limitations of PEGylation.

Keywords: Chitosan, chitosan nanoparticles, protein corona, chitosan derivatives, immune system

Teaser: The development of strategies, beside PEGylation, to reduce the formation of protein corona on chitosan-based nanoparticles is a key step towards their biomedical applications.

Chitosan is a co-polymer of β-(1,4) linked D-glucosamines and N-acetyl–D-glucosamines, derived from the alkaline deacetylation of chitin, a widespread natural polymer, mostly found in bacteria, fungi and in the exoskeletons of crustaceans and insects [1]. Chitin and its derivatives are used extensively in medical practice due to their non-toxic, biocompatible and non-allergenic properties [1]. The immunological activity of chitin was discovered in the 1980s, when it was demonstrated that chitin and chitosan can lead peritoneal macrophages and natural killer cells to produce pro-inflammatory cytokines such as interferon gamma (IFNγ), interleukin-1β (IL1β) and colony stimulator factor (CSF) for viral and tumour protection [2]. Interestingly, these effects were found to be more efficient if the material was a mixture of 70% chitosan and 30% chitin instead of the corresponding pure forms [1]. Moreover, as an adjuvant in vaccine formulation, chitosan encouraged the intracellular release of DNA by mitochondria in vitro, activating the stimulator-of-interferon-gene (STING) pathway to produce type I interferon (IFN), which plays a key role in antiviral immunity. Hence, chitosan was responsible for dendritic cell maturation followed by cell immunity induction [3].

The degree of deacetylation (DDA) and the molecular weight (MW) of chitosan depend on the extent of alkaline deacetylation of chitin, so experimental parameters such as reaction time and concentration of base are determining factors. The DDA of chitosan determines the proportion of free primary amino groups on its backbone and is responsible for biodegradability properties: at high values of DDA (>90%) chitosan is slightly cytotoxic and non-biodegradable [4], whereas at values of DDA ranging from 50% to 85%, chitosan is degraded by macrophages and neutrophils [4]. The MW of chitosan also determines the extent of in vivo biodegradability. Indeed, it was reported that high MW chitosan degraded slowly in vivo, increasing the chances to accumulate in the tissues following repeated administrations [5,6]. The DDA is also responsible for chitosan's mucoadhesive property. This is of paramount importance for drug delivery purposes since, upon slightly acidic environment, the positive amino groups on chitosan interact with the negatively charged cell membrane, increasing the retention time and the absorption of the nanocarrier (Figure 1) [7]. Moreover, it has been shown that the free positive amino groups of chitosan can interact with plasma proteins or blood cells, inducing a thrombogenic and/or haemolytic response, through complement system activation and blood coagulation [8]. Comprehensive reviews concerning the haemocompatibility of chitosan materials have been provided by Balan et al. [9] and, more recently, by Hu et al. [10].

Chitosan is widely employed in the production of drug delivery systems such as micelles, liposomes and nanoparticles (NPs) [11]. NPs have received considerable attention, owing to their ability to overcome many hurdles that the free therapeutic agents encounter upon systemic administration, such as poor solubility, low bioavailability and fast degradation in biological media [12,13]. Instead, NPs confer high drug's therapeutic efficacy, fine control of its release and protection from enzymatic degradation [14]. However, when dissolved in biological fluids such as plasma, NPs promptly adsorb proteins onto their surface [15]. This physisorption event leads to the formation of a "protein corona" impairing the targeted delivery process of the drug, hence its intended therapeutic efficacy. A recent multivariate analysis on the efficiency of NP's delivery revealed that only the 0.7% of administered NPs could reach the target solid tumour in mouse models [16]. Indeed, hundreds of proteins are adsorbed on the surface of NPs and, according to their nature, the systemic circulation time of NPs can be compromised [15].

The reticuloendothelial system (RES) represents the first barrier that nanotechnology's therapeutic design needs to overcome [17]. Indeed, upon systemic administration, most NPs fail to reach the target since they are captured by the RES, which is part of the immune system: the RES is constituted by phagocytic cells of the reticular connective tissue, composed of collagen and located around the kidneys, liver, spleen and lymph nodes. In vivo, most NPs are subject to two possible routes of clearance: the kidneys or the mononuclear phagocyte system (MPS). The latter comprises all organs possessing macrophages, such as lymph nodes, liver, spleen, bone marrow and skin [17,18]. However, most of the NPs are sequestered by the liver and spleen [18]. The method of excretion depends on the size of NPs: those whose size is suitable for glomerular filtration (< 6 nm), are eliminated by the kidney; larger NPs are captured by Kupffer cells in the liver and pass through the bile to the faeces [19].

The protein corona on NPs represents the real surface that will interact with the biological barrier, and this interaction is known as the "nano bio interface" [20]. The protein corona determines the fate of the nanocarrier inducing the uptake by the MPS. Indeed, the nature of proteins that bind to the surface of NPs can lead to opsonisation, where NPs are surrounded by opsonins such as fibronectin, components of the complement system and immunoglobulin G (IgG). Opsonins act as markers for phagocytosis, binding to the scavenger receptors on macrophages of the RES, hence inducing accumulation in the liver and spleen [21,22]. This process of opsonization can be used to target these organs. NPs can otherwise be coated with other proteins called dysopsonins (e. g. glycoprotein CD47, albumin or apolipoproteins), which inhibit phagocytosis. In this case, NPs bypass the macrophageal clearance, showing prolonged bioavailability (Figure 2) [21,22].

Protein coronae are classified as "soft" or "hard", according to how strongly proteins are adsorbed on the NPs surface. High abundant, fastest binding and lower MW proteins (such as albumin and globulins) will form the initial or "soft" corona, which lasts from seconds to minutes [21]. Then, according to the Vroman effect these proteins are replaced by higher affinity and higher MW proteins (such as coagulation factors and apolipoproteins), resulting in the "hard" corona, which can take a variable time to form (up to several hours) according to the physicochemical properties of the nanocarrier (Figure 3) [23]. Hence, the hard corona is responsible for the fate of the NPs: inducing the immunological response or providing a reduction in toxicity [21].

The protein corona can change the physicochemical properties of NPs including the hydrodynamic size (which usually increases), aggregation attitude and surface energy or zeta potential (ZP) [22]. At physiological pH, most serum proteins are composed of negatively charged amino acids which can interact with positively charged nanocarriers [22]. For instance, chitosan nanoparticles (CtNPs) can interact with the negatively charged albumin, which is found in high concentration in the blood plasma [24]. Albumin is known to be a dysopsonin, hence providing a stabilizing effect on NPs [25]. However, adsorption of serum proteins can cause severe aggregation and clearance of NPs with impact on their in vivo application [26]. Zubareva et al. [27] assessed the fate of positive hydrophobicly-modified hexanoyl CtNPs (HCtNPs) and negatively charged succinoyl CtNPs (SCtNPs) in murine macrophage-like RAW264.7 cells: HCtNPs were taken up more efficiently than SCtNPs (Figure 4). Moreover, the effect on the size and ZP of NPs upon incubation in complete cell culture medium supplemented with 10% fetal bovine serum (FBS) was studied. Since the concentration of albumin in the serum caused the charge of the medium to be around –20 mV, incubation of HCtNPs in the medium for 30 min induced the ZP to decrease from +35 mV to -13 mV, while increasing the size by 20%. In contrast, under the same incubation conditions, SCtNPs maintained the negative ZP (-28 mV) accompanied by a size decrease of 30%. This effect had previously been reported in the literature [28]. Understanding how NPs interact with the biological milieu is key to develop suitable tumour targeting drug delivery systems [29]. On this regard, several reviews focusing on the improvement of the design of nanocarriers [23,30–32] along with analysing the effect of protein corona on theragnostic

applications of nanocarriers [33] have been recently published. Especially, a recent review shed lights on the factors, such as physico-chemical properties that affect the formation of protein corona on CtNPs and its impact on drug delivery applications [34].

Owing to the presence of positively charged primary amino groups, CtNPs are highly affected by the formation of corona inducing phagocytic uptake and clearance. Therefore, surface functionalisation of CtNPs is needed to prolong NPs circulation time. This review offers a discussion of strategies developed in the last 15 years to increase the in vivo biodistribution of chitosan-based nanocarriers, overcoming the limitations shown by PEGylation (Table 1, Figure 5). Moreover, advantages and disadvantages of those strategies are discussed so to understand future research direction.

Materials for coating chitosan nanoparticles

Polyethylene glycol

Cationic NPs made of polymers such as chitosan are being extensively investigated for nucleic acid-based therapies. However, their intravenous (IV) administration leads to significant in vivo toxicity, limiting their clinical application [35]. Coating the surface of NPs with polymeric hydrophilic neutral materials was beneficial in several ways, such as increasing their stability and solubility in neutral milieux [36]. Polyethylene glycol (PEG) can be adsorbed or covalently bound to the surface of CtNPs [37] to achieve long term blood circulation [37,38]; PEG coating (PEGylation) increases the water solubility of chitosan, avoiding CtNPs to precipitate in solutions at pH > 6.5 [39]. Hence, PEG co-polymerization with chitosan can increase the physical stability and blood circulation time of NPs [39–43]. PEGylation is a useful method to improve the gene transfection of CtNPs by increasing the stability of the gene loaded CtNPs. In a study by Rastegari et al. [44], chitosan lactate NPs were coated with PEG to improve their stability during the delivery of small interfering RNA (siRNA) to treat diabetes. In vivo studies were carried out on rats treated with siRNA-loaded PEGylated NPs. PEGylation induced higher stability of siRNA in the blood by decreasing the interaction of NPs with the blood components and impeding enzyme degradation [44]. Furthermore, Li et al. [45] studied the protection of plasmid DNA (pDNA) by PEGylated CtNPs under incubation with DNase. The resistance of pDNA toward the DNase was enhanced when the pDNA was encapsulated in PEGylated NPs, compared to free pDNA or pDNA loaded in uncoated CtNPs [45]. Ragelle et al. [39] assessed the stability of siRNA-loaded PEGylated CtNPs, and found that the NPs did not dissociate in the plasma since a 100% complexation efficiency was recorded.

However, a “PEG dilemma” has emerged since the coating showed limitations in the interaction and internalization of the carrier at the cellular level of the targeted cells, affecting drug delivery [46]. Moreover, anti-PEG antibodies can be produced, activating the complement cascade and reducing the drug’s therapeutic efficiency [47] (Figure 6). Finally, an elaborate procedure and organic solvents are needed for the covalent PEG conjugation. Therefore, alternatives to PEGylation needed to be implemented.

Carboxylated PEG (100) monostearate and D tocopheryl PEG (1000) succinate

Wang et al. [48] coated chitosan NPs with two amphiphilic derivatives of PEG, carboxylated PEG (100) monostearate (PEGms) and D-α-Tocopherol PEG (1000) succinate (tPEGs), by a non-covalent method. Such method resulted in a detachable coating [49] that did not interfere with the cellular uptake: CtNPs were able to deliver genes efficiently to the tumour when PEG was non-covalently bound [50]. Polyelectrolytes complex NPs were formed based on hyaluronic acid (Ha) and chitosan hydrochloride (CtHc). NPs were loaded with mitoxantrone, a widely used anti-cancer drug but fraught with many side-effects when administered in its free form [51]. The protein adsorption by NPs was then evaluated in vitro upon incubation of formulations with bovine serum albumin (BSA) solution; unbound proteins were quantified by the Folin reagent method. It was found that both formulations (NPs coated with PEGms or tPEGs) showed a similar protein binding (about 30%), whereas the protein binding for uncoated NPs was significantly higher. The protein adsorption was also measured in terms of size of NPs: the size of uncoated NPs increased upon incubation with BSA; on the other hand, no size increment was observed when NPs were coated with PEGms or tPEGs. These results suggested that both PEG derivatives were able to decrease the adsorption of plasma proteins, indicating a long lifetime of NPs in vivo. However, cellular uptake mechanisms in this study were only addressed to assess the receptor-mediated endocytosis given by the HA towards the CD44 receptor widely expressed on cancer cells [52]. No effect of the discarded coating on the cellular uptake was evaluated, and further studies are needed.

Methoxypolyethyleneglycol

Methoxypolyethyleneglycol (mPEG) has been used to modify the surface of chitosan micelles, reducing the formation of protein corona and acting as a carrier of paclitaxel [53]. Indeed, it has been found that mPEG coating could increase the micelles' stability in the blood and lower their uptake by the RES [54]. The structure of chitosan was also modified with cholesterol and N-acetyl L-cysteine in order to obtain spontaneous micelle formation in an aqueous medium [53]. To assess the adsorption of serum proteins, mPEG-coated chitosan micelles were incubated in BSA: the coating with mPEG considerably decreased (13.8%) the protein adsorption compared to uncoated chitosan micelles (59.3%). As a result, it was deduced that the circulation time of mPEG-coated chitosan micelles could be extended, in turn increasing the bioavailability of the cargo [53,55,56]. Indeed, the antitumour potential of paclitaxel-loaded mPEG-coated chitosan based micelles was investigated in an in vivo tumour regression study and a significant reduction in tumour size was observed [53]. From this study, one can deduce that mPEG is a safer and more efficient derivative of PEG. However, the production of antibodies against these derivatives of PEG employed to coat chitosan nanocarriers was not tested, to effectively demonstrate that the PEG dilemma had been overcome.

Carboxymethyl dextran

Tekie et al. [57] considered the formation of chitosan-based polyelectrolyte complexes as an easier alternative to chitosan polymerization with PEG. Stable polyelectrolytes can be easily obtained in aqueous media by electrostatic interactions between oppositely-charged polymers, such as chitosan and carboxymethyl dextran [58]. Polyelectrolytes with different surface properties were prepared by altering the

dextran/chitosan molar ratio, since protein corona is affected by NPs' surface properties [59]. The protein corona formation was assessed in chitosan-based polyelectrolytes by incubating NPs with FBS, followed by removal of the soft corona (NPs were isolated by centrifugation at 13000 rpm, followed by washing with PBS [60]). The size of NPs significantly increased at low dextran/chitosan ratio, whereas not much variation in size was recorded at high dextran/chitosan ratio, indicating that the presence of dextran reduced the adsorption of serum proteins on NPs. A possible reason was that the carboxylate groups, which conferred negative charges to the nanocarrier, increased the repulsion of negatively charged proteins. The protein corona was analysed by LC MS/MS spectroscopy and found to be composed mainly of protein C, haemoglobin and apolipoproteins. In presence of FBS, the small amount of protein corona in chitosan/dextran polyelectrolytes induced a high cellular uptake of NPs in a breast cancer cell line, also partly due to an increase in cell function owing to FBS. Biodistribution studies showed a long circulation time of the NPs, suggesting that chitosan/dextran polyelectrolytes could provide an efficient drug delivery system [57]. Further studies may include the incorporation of a chemotherapeutic drug such as doxorubicin and testing in tumour-bearing mice, to investigate the effective delivery of the drug to the target in terms of tumour mass reduction.

Hyaluronic acid and alginate

Almalik et al. [61] formed CtNPs via ionic gelation and then coated them with anionic polysaccharides such as hyaluronic acid (HA) or alginate (Alg). HA is a biodegradable and biocompatible glycosaminoglycan found in extracellular matrices and is extensively used to target tumour cells (such as colon cancer cells) overexpressing CD44, the HA receptor. This is also found on inflammatory cells and, indeed, RAW 264.7 macrophages were able to engulf CtNPs coated with HA by a receptor-mediated mechanism [62,63]. Regarding the size, HA coating formed negatively charged NPs with increased diameter due to agglomeration during the coating process, whereas Alg coating produced more strongly charged NPs due to the higher charge of Alg, resulting in increased size. The protein corona formed upon interaction of NPs with serum proteins was then analysed. Uncoated CtNPs showed high reactivity to serum proteins resulting in a dense protein corona, while HA coating led to lower protein adsorption on the surface of NPs. Indeed, when analysing the protein corona by mass spectrometry, each type of NP showed different adsorption of proteins. The proteomic profiles showed that uncoated CtNPs adsorbed 18 proteins, HA coating induced adsorption of 16 proteins while Alg coating led to the adsorption of 25 proteins. When looking at protein identities, the Alg coating produced a broader spectrum of adsorbed proteins (14 proteins) than uncoated CtNPs (3 proteins) and HA-coated CtNPs (5 proteins). This could be due to the larger size of Alg-coated NPs, which induces a larger number of proteins to interact with each NP [64]. The nature of proteins was mapped by gene ontology and showed that HA coating decreased the adsorption of proteins involved in inflammatory activities such as the complement pathway, suggesting that HA coating led to less immunogenic NPs compared to uncoated or Alg coating. HA-coated CtNPs were predominantly coated by two anti-inflammatory proteins, alpha 1 acid glycoprotein (AGP) and inter alpha trypsin inhibitor heavy chain H4 (ITIH4). On the other hand, both CtNPs

and Alg-coated CtNPs adsorbed clusterin, a proinflammatory apolipoprotein linked to immune regulation, cell adhesion and active cell death [65]. In this way, immune-modulating nanomaterials with immune activating or suppressing surfaces can be prepared [61]. Since the presence of HA on the surface of CtNPs confers tumour-targeting properties, HA-coated CtNPs can be a valuable tool in drug delivery and further biodistribution studies may help to understand their fate.

Hyaluronic acid and aptamers

Varnamkhastia et al. [66] generated NPs of appropriate size and charge in a core-shell manner: the positive core was constituted by chitosan interacting with HA, which was the negative shell. HA was conjugated through an ester bond to SN 38, a highly hydrophobic active metabolite of the chemotherapeutic agent irinotecan. The conjugation avoided the conversion of SN 38 to its inactive metabolite and increased its stability. Finally, DNA aptamers were employed as targeting moieties and designed against the tumour marker MUC1, a mucin glycoprotein overexpressed in several epithelial cancer cells such as colon cancer cells [67]. Since aptamers have a low MW (6 - 24 kDa), the RES barely recognized them, allowing NPs to have a longer circulation time [68]. The formation of a protein corona on NPs was evaluated following their incubation with either 10% or 100% FBS. SDS-PAGE results indicated that a significantly higher amount and range of proteins were adsorbed on NPs incubated with 100% FBS (due to the high concentration of serum proteins) than 10%. Moreover, NPs incubated in FBS showed increased size (from 125 nm to 151 nm) and more negative surface charge (from +14.5 to -5 mV) compared to NPs before incubation. Since NPs incubated with 100% FBS simulated the in vivo protein corona behaviour, they were incubated in a free serum medium to assess their cytotoxic activity against a human colon cancer adenocarcinoma cell line (HT29) and it was found that the protein corona of the modified NPs had no impact on cytotoxicity. However, flow cytometric analysis showed a decreased uptake of aptamer modified NPs incubated with 100% FBS by the HT29 cell line, due to the formation of a protein corona: the corona coating decreased the interaction with the cell receptor, and hence the targeting efficiency [66]. Therefore, coating with an aptamer is not helpful in overcoming the protein corona effect in CtNPs, especially in terms of NPs-cell interactions.

Polyacrylic acid

Wu et al. [36] employed the polyanionic poly(acrylic acid) (PAA) to coat cationic CtNPs formed by ionic gelation. PAA was conjugated to the surface of the NPs by water-soluble carbodiimide (EDC): advantages included high stability in plasma due to negative surface charge, integrity of the hydrophobic core of CtNPs with no impact on drug loading, finally availability of functional groups on chitosan for further derivatisation [36]. The stability of PPA- CtNPs in serum was assessed by incubating fluorescein isothiocyanate (FITC)-BSA with CtNPs and investigating their behaviour towards protein adsorption. The intensity of unbound FITC-BSA in the supernatant was measured following centrifugation of the NPs and found to be 15.3% higher than the control (uncoated NPs), indicating that PAA-NPs were more resistant to protein adsorption owing to their negatively charged surface. However, the surface charge of PAA-NPs was found to attenuate the cellular uptake due to electrostatic repulsion with the negatively charged cell membrane. Therefore, in

order to increase cell internalization, other functionalisation strategies of CtNPs must be adopted beside PAA-coating, such as conjugation with a ligand that interacts with a receptor on tumour cells [36]. Moreover, the extent of the effect of the decreased protein binding should be further investigated in macrophage cellular uptake studies, so to assess whether PAA-CtNPs can be engulfed by the macrophages of the RES, and their blood circulation time could effectively be prolonged.

Polymethacrylic acid

Zhang et al. [69] coated the surface of CtNPs with the polyanion polymethacrylic acid (PMA) to enhance their stability in the bloodstream. In vivo experiments were performed on Sprague Dawley mice by injecting solutions of NPs coated or not with PMA. To determine their concentration in the plasma, NPs were fluorescently labelled with FITC. At different time points, blood was withdrawn, and the plasma separated. The concentration of PMA-coated NPs in the plasma was assessed by fluorimetry and found to be higher than uncoated NPs over time, indicating a prolonged lifetime of coated NPs in the bloodstream. This was also confirmed by measuring the blood clearance of NPs, which was significantly slower for PMA-coated NPs (6.72 mL/h) than uncoated (30.57 mL/h) [69]. Finally, the chemotherapeutic agent, 10 hydroxy camptothecin, was incorporated in the NPs and the in vivo antitumour efficacy was assessed in tumour-bearing mice. The data showed that the tumour growth of mice treated with drug loaded PMA coated NPs decreased compared to the control (unloaded drug). Overall, the results by Zhang et al. [69] are encouraging for further clinical investigation.

Chitosan derivatives as backbone of nanocarriers

Chitosan imidazole-acetic acid derivative

Toy et al. [70] investigated the mechanisms of CtNP's in vivo systemic toxicity by modifying the free amino groups on chitosan with imidazole-acetic acid (IAA). This modification resulted in the tools like receptor 4 (TLR 4) downregulation in macrophages [71]. Indeed, the in vivo systemic toxicity of chitosan primary amino groups is due to the activation of both TLR 4 in macrophages and complement system. Activated macrophages, in turn, secrete tumour necrosis factor alpha (TNFα) and initiate the innate immune response [72]. The resulting IAA-modified CtNPs had a slightly positive surface charge (ca. +15 mV) at physiological pH. Following IV administration of NPs to mice, serum analysis of proinflammatory cytokines (TNFα, IL 6 and IL 1β) showed that IAA-modified CtNPs induced lower cytokine levels in the serum than unmodified CtNPs, indicating that IAA was effective in reducing the innate immune response. However, IAA-modified CtNPs induced in vivo complement activation and showed higher neutrophil uptake than unmodified CtNPs. Moreover, following exposure to serum proteins ex vivo, it was found that chitosan modified with IAA reduced complement adsorption, a phenomenon that normally occurs after 1 h incubation of unmodified CtNPs with mouse plasma [70]. This study concerns in vitro cytotoxicity screening of NPs and is key to understanding the in vivo toxicity of cationic polymer NPs such as CtNPs for future clinical applications. Functionalization of CtNPs with IAA was effective in decreasing the activation of the innate immune

response. However, biodistribution studies carried out by Toy et al. [70] showed that IAA modified CtNPs accumulated mainly in liver, kidney and lung, indicating elimination of NPs by macrophages of the RES. Therefore, further studies are needed in order to prolong the in vivo blood circulation time of CtNPs.

Chitosan ornithine derivative

Ornithine derived by the breakdown of arginine in the human system and is known to enhance the cellular uptake of cancer cells in transfection studies [73]. Ornithine was chemically grafted onto low MW chitosan backbone (previously showed to enhance transfection efficiency [5]) and the adsorption of plasma proteins by the conjugate was investigated by PAGE gel electrophoresis [74]. Two amounts (50 and 100 μg) of polymer were incubated with human plasma diluted with Phosphate Buffered Saline (PBS) and found that the protein adsorption was negligible in both cases, indicating a low risk of immunological response. Further, semi-quantitative densitometry analysis of the gel image was performed: at high concentrations of polymer, a slight reduction of protein adsorption occurred, corresponding to the adsorption of albumin. The polymer was then condensed with plasmid DNA, previously conjugated with YOYO iodide and the cellular uptake was assessed in a murine glioma cell line in presence of FBS [74]. Fluorescence images showed high intensity in the intranuclear region, suggesting that the presence of ornithine enhanced the cellular uptake, especially nuclear uptake. Results of this study are promising but the protein adsorption was evaluated only on the polymer and not on the polymer condensed with plasmid DNA, forming nanocomplexes whose chemical-physical characteristics may differ. Moreover, no in vivo biodistribution study was conducted to establish a correlation between the presence of ornithine on the nanocomplexes and the circulation time of the gene delivery system.

N-phosphorylcholine chitosan derivative

In a study by Wang et al. [75], low MW chitosan was linked to N-phosphorylcholine (Pc) by a phosphoramide bond [76], so as to obtain a biomimetic chitosan-based NP that could avoid interaction with plasma proteins. Pc is an electrically neutral head group, representing the main constituent of the external membrane of erythrocytes and was chosen due to its haemocompatibility and resistance to protein adsorption [77]. NPs were prepared by ionic gelation and then interactions between NPs and BSA, a model protein, were analysed by UV absorption and fluorescence emission spectrometry. The results showed that BSA underwent a conformational structural change when interacting with the control, but no change was recorded upon incubation with Pc-CtNPs, suggesting no interaction was taking place. The authors [75] also found that the size and the ZP of Pc-CtNPs (90.4 ± 1.3 nm; +14.2 ± 0.8 mV) were lower than those of the control (157.4 ± 3.2 nm; +43.4 ± 1.4 mV). A lower ZP value of Pc-CtNPs might induce reduced interactions with BSA; however, more studies such as biodistribution investigations are needed to establish that this biomimetic chitosan is effective in prolonging the circulation time of CtNPs in the blood.

N-octyl-N, O-carboxymethyl chitosan

Huo et al. [6] assessed the in vivo blood stability of micelles made of N-octyl-N,O-carboxymethyl chitosan, an amphiphilic derivative of low MW chitosan. Chitosan was conjugated to fluorescein isothiocyanate (FITC) and micelles were intravenously administered to healthy mice. The concentration of fluorescent micelles was found to be higher in the plasma (~ 85% of the dose), than the liver (~ 10%) or other organs (~ 0.5%). The high concentration of micelles in the plasma suggested that this polymer generated micelles that were stable in the blood circulation and characterized by a very low accumulation in the MPS organs for their removal. In this study, micelles had a highly negative ZP (approximately -30 mV) due to the ionized carboxyl groups on their surface. This was advantageous as very low interaction with tissues and blood proteins was recorded. However, it may also be disadvantageous as chitosan with a strong negative surface may be toxic and lose the properties associated with its positively charged surface such as the mucoadhesive property. On this regard, the same group [78] recently assessed the mechanisms of oral absorption of N-octyl-N,O-carboxymethyl chitosan-based micelles in a widely used intestinal epithelial cell line (Caco-2 cells). Results showed that several mechanisms such as tight junctions opening are involved in the transport of micelles across the barrier. In addition, toxicity studies showed high biocompatibility of this polymer. However, this recent study did not focus on the IV administration of micelles followed by tumor targeting abilities. Hence, possible future investigations may assess reduction in tumor size following IV administration of anti-cancer drug loaded N-octyl-N,O-carboxymethyl chitosan-based micelles.

Chitosan oligosaccharide

It was shown that converting chitosan to oligomers characterized by higher water solubility was advantageous for its immunostimulant, anti-inflammatory and anti-tumour properties. Chen et al. [79] demonstrated the immunomodulatory response of water-soluble low MW chitosan due to macrophage activation in the context of allergic asthma.

Chitosan oligosaccharide (CtO), a low MW product of chitosan depolymerization [80], was employed by Zhang et al. [81] to prepare self-assembled NPs by hydrophobic modification with oleic acid. The adsorption of proteins by NPs was assessed upon incubation with bovine calf serum (BCS) containing BSA. The proteins were then desorbed from the NP surface and applied to SDS PAGE. Upon incubation, proteins adsorb onto the NPs in a concentration- and time-dependant manner. When examining the corona composition, the main protein found was BSA, adsorbed on the NPs in a time-dependent manner. This was found to be beneficial for CtONPs as the stability of BSA adsorption indicated protection against NPs degradation, with prolonged blood circulation time [81]. Nevertheless, the BSA adsorption may interfere with the cellular uptake after in vivo drug delivery. Zhang et al. [81] assessed the cellular uptake in a human lung carcinoma cell line which was cultured in the presence of BCS. However, BSC was removed upon incubation with NPs, hence the effect on the cellular uptake of NPs coated with albumin was not clearly investigated and further studies are needed.

Chitosan as stealth coating of nanocarriers

Carboxymethyl chitosan

Carboxymethyl chitosan (CmCt) shows higher solubility and biocompatibility than chitosan [82], overcoming one of the major limitations of chitosan for biomedical applications, namely its poor solubility in physiological milieux. CmCt, being negatively charged, was used as a protective shell for liposomes which were previously coated with a cationic polymeric layer constituted by trimethyl chitosan [83]. Indeed, the stability and biocompatibility was shown to be further improved by this system [84], hence it was used for the oral delivery of curcumin [83]. The CmCt coated liposomes were incubated with BSA to allow protein adsorption. Proteins were then desorbed from the liposomes surface, finally analysed by SDS PAGE and Bradford method for quantification. CmCt coated liposomes showed lower protein adsorption (3.3%) compared to liposomes coated only with trimethyl chitosan (5.9%). Biodistribution studies on rats showed that CmCt-coated liposomes accumulated mainly in the spleen, liver and lungs owing to macrophages of the RES located in these organs [83,85,86] Conversely, the control (liposomes coated only with trimethyl chitosan) concentrated mainly in the kidneys for elimination. These results indicate that CmCt-coated liposomes were able to increase the oral bioavailability of curcumin and its retention time in the blood circulation. However, they mainly accumulated in the RES organs, which indicates elimination of the drug carrier. Hence, further studies are needed to reduce the uptake of the CmCt-coated liposomes by the macrophages of the RES, especially if the drug is meant to be delivered to other organs, such as the brain.

Chitosan oligomers

To overcome the “PEG dilemma”, Amoozgar et al. [87] employed low MW chitosan ranging from 2 to 6.5 kDa to covalently coat the surface of NPs made of polylactic-co-glycolic acid (PLGA) through the formation of an amide bond [87]. Low MW chitosan coating imparted hydrophilic properties to the surface of NPs. Therefore, the ability to enable pH-sensitive cell interactions (so as to deliver drugs specifically in the weakly acidic tumour microenvironment) was assessed [87]. Following incubation of NPs with 50% FBS, gel electrophoresis studies showed that low MW chitosan coated NPs had lower protein adsorption (ranging from 18 to 42 μg/mg) than uncoated NPs (68 μg/mg), but still higher than PEG-coated NPs (3.9 μg/mg). The cellular uptake by J774A.1 macrophage was also evaluated by labelling PLGA with fluoresceinamine and analysed by confocal microscopy. A similar trend was observed: a smaller uptake was recorded for low MW chitosan-coated NPs than uncoated, but still greater than that of PEG-coated NPs. However, there was fast drug release in the first 24 h, and hence little promise for tumour-specific drug delivery [87]. The same group later demonstrated that coating NPs with low MW chitosan by the dopamine polymerization method could overcome the issue of premature drug release. Indeed, through the resulting polydopamine layer, low MW chitosan was incorporated into the PLGA-NPs, conferring greater ability in retaining the drug. Incubation of NPs with 50% FBS induced the surface of NPs to be surrounded by a hard corona, consisting mainly of albumin and IgG, which are abundant in FBS. Surprisingly, a study of the interaction of NPs with J774A.1 macrophages revealed that the layer of low MW chitosan could reduce phagocytic uptake regardless of the protein binding, even at greater extents of PEG coating [88]. Similarly, a NP cell interaction study was

carried out using a human ovarian tumour cell line. Results showed an efficient cellular uptake of those NPs, suggesting that uptake was not affected by the protein corona [88]. However, the surface of NPs needs to be optimized before carrying out in vivo investigations, since the adsorption of IgG on NPs surfaces indicates activation of the humoral immunity leading to NPs elimination in vivo.

Zwitterionic chitosan

Xu et al. [89] assessed low MW chitosan conjugated with succinic acid for its ability to avoid opsonization and be suitable for in vivo applications. Chitosan was functionalized by reaction with succinic anhydride to form an amide on the primary amine groups, resulting in a pH-sensitive "zwitterionic" polymer, characterized by increased water solubility, anionic surface at physiological pH and positive surface in acidic environment. This allows its detachment from the coated surface upon acquisition of positive charges in the tumour microenvironment, leaving the nanocarrier free to interact with the tumour cell, overcoming one of PEG's limitations. As a preliminary study, zwitterionic chitosan was employed to coat the cationic surface of Eudragit E100, a polymeric film that would attract anionic proteins. Protein adsorption was then quantified following incubation with BSA or FBS at pH 7.4. The coating with the zwitterionic chitosan reduced BSA adsorption by 75% and FBS adsorption by 43%, as was shown by Cao et al. [90]; moreover, zwitterionic chitosan was found to be haemocompatible. Finally, complement activation was assessed and found to be significantly lower in presence of zwitterionic chitosan compared to unmodified chitosan. Altogether, these results showed that zwitterionic chitosan can overcome the disadvantages of PEG, and can be safely employed for the coating of tumour targeting cationic drug delivery systems [89]. For instance, the anionic feature of zwitterionic chitosan at physiological pH has been employed to coat the surface of polyamidoamine (PAMAM) dendrimers, widely used for gene delivery owing to their cationic surface but potentially cytotoxic and prone to cause opsonization [91]. It was shown that the coating successfully protected healthy cells from the toxic effects of PAMAM dendrimers at physiological pH, but left them free to interact with cancer cells upon detachment [91]. In another study, the zwitterionic chitosan functionalised with succinyl groups was further modified with both long alkyl chain and folate to confer hydrophobicity and tumour-targeting specificity, respectively [92]. This new polymer was employed in the formulation of micelles as tumour-targeting drug delivery systems whose efficacy was evaluated both in vitro and in vivo and shown to have considerable potential for use in chemotherapeutics [92]. Ju et al. [93] developed a nanogel based on succinyl-modified chitosan, to allow large NPs to penetrate tumours via the ability of the gel to swell and shrink upon pH variation. Following swelling, NPs were released and the encapsulated tumour agent delivered to cancer cells [93]. Overall, these studies suggest that zwitterionic chitosan can be a suitable polymer that overcome PEG's limitations in terms of NP-cells interaction.

Discussion

In the last decade extensive research has been dedicated in the generation of drug delivery systems, using biocompatible nanocarriers such as those based on chitosan. However, the success of a designed nanocarrier in delivering therapeutic agents to the target site may be impaired by in vivo conditions. Indeed, upon IV

administration, nanocarriers promptly interact with macromolecules found in the blood forming a protein corona that plays a key role on the fate of the nanocarrier in the body. Indeed, according to the type of proteins adsorbed on the surface of the nanocarrier, its retention time in the blood can be prolonged or reduced with impact on its targeted delivery [30].

The development of nanocarriers or coatings able to escape the immune system is key for biomedical applications. The strategies applied to chitosan-based NPs aim to overcome the "PEG dilemma" conferring on them the ability to prolong the retention time in the blood and to keep the targeting ability. Indeed, nanocarriers based on pristine chitosan are susceptible to the adsorption of plasma proteins due to the presence of positively charged amino groups on the chitosan backbone. In addition, the amino groups may cause systemic toxicity due to the activation of TLR4 in macrophages and complement system [72]. The general approach is to mask the positive charges on CtNPs, either by coating them with anionic polymers or by modifying the amino groups with functional molecules. Both approaches may affect the mucoadhesive property of chitosan, hence its ability to perform ionic interactions with the negatively charged cellular membrane.

Recently, much research has been done in escaping the immune system upon in vivo conditions by exploiting the protein corona, using a preformed protein corona or biomimetic protein corona on NPs [15,94]. For instance, coating of NPs with endogenous peptides such as CD-47 (an integrin-associated protein) was shown to increase the circulation time of NPs by avoiding macrophage uptake [95]. For the purpose of forming biomimetic CtNPs and conferring resistance to protein adsorption, chitosan was conjugated to Pc [75] or modified with ornithine [74]. Indeed, the biomimetic coating helps NPs to evade the immune system recognising them as self. Moreover, it confers on NPs improved targeting abilities while reducing off-target effects. For instance, Zhang et al. [96] achieved brain targeting effect by modifying the surface of liposomes with a short biocompatible peptide $A\beta_{1-42}$. This peptide specifically adsorbed plasma apolipoproteins A1, E and J, whose receptor is expressed on the blood-brain barrier, hence facilitating the internalization by receptor-mediated transcytosis. Regarding chitosan, adsorption of apolipoproteins for brain targeting has been achieved by coating NPs with polysorbate 80 [97]. However, this coating may not be suitable if the target is not the brain. Indeed, apolipoproteins are regarded as a component of the hard corona which may reduce cellular interactions if the target cell does not express a receptor for apolipoproteins.

Upon in vivo condition, its high concentration in the blood induces the negatively charged albumin to be the main component of protein corona on positively charged CtNPs, due to electrostatic interactions. Albumin (considered a dysopsonin) may be beneficial on the surface of CtNPs. Indeed, it was found a high interaction between albumin coated NPs and endothelial cells, in turn prolonging their circulation time [94]. Conversely, Nguyen et al. [98] found that precoating of gelatin-oleic NPs with albumin increased the cellular uptake of a human embryonic kidney cell line (HEK 293) while decreasing that one of a human adenocarcinoma alveolar basal epithelial cells (A549). This result suggests that albumin coating is not always advantageous, due to the loss of targeting effect. Albumin coating on the surface of CtNPs would lead to the loss of the

mucoadhesive property of chitosan along with the ability of releasing the drug to the target site. Furthermore, albumin is regarded as a low MW protein hence a component of the soft corona. Although it may have high affinity with the positive surface of chitosan, according to the Vroman effect, other proteins at higher MW may form the hard corona on pristine CtNPs, limiting their blood circulation time and targeting efficiency. The general method adopted by the literature to analyse the degree of protein adsorption is by incubating the nanocarrier with BSA or FBS. This indicates that the aim of any possible strategies applied to chitosan-based NPs is to decrease proteins adsorption, especially of albumin. Therefore, further investigations would be beneficial to understand at which extent albumin coating may help CtNPs in their drug delivery mission.

Some strategies aimed to overcome the "PEG dilemma" by coating CtNPs with materials beyond pristine PEG and albumin adsorption was tested. To start with, PEG derivatives (PEGms, tPEGs [48] and mPEG [53]) have been shown to overcome the PEG dilemma by providing a detachable coating that does not interfere with the targeting ability of the nanocarrier. The coating of CtNPs with these derivatives resulted in a lower protein binding when compared to uncoated CtNPs, suggesting a longer retention time upon in vivo conditions. However, the level of anti-PEG antibodies was not assessed upon IV administration, hence further studies are needed.

Other materials were used to coat the surface of CtNPs so to reduce protein adsorption. These include mainly anionic materials such as carboxymethyl dextran [57], hyaluronic acid [61], alginate [61], polyacrylic acid [36] and polymetacrylic acid [69]. The use of aptamers [99] was also investigated. Carboxymethyl dextran coating induced low adsorption of proteins along with high cellular uptake and long circulation time of NPs [57]. These results suggested that carboxymethyl dextran could be a suitable alternative to PEG, though the loading of a chemotherapeutic drug may further be investigated. Hyaluronic acid coating resulted in less immunogenic NPs compared to alginate coating [61] and its tumour targeting ability may be further explored in vivo. The negative surface charges conferred by the coating with polyacrylic acid or polymetacrylic acid were advantageous in terms of protein adsorption. However, results from Wu et al. [36] were less encouraging than Zhang et al. [69] since the negative coating was found to interfere with the cellular internalization of the nanocarrier, hence further functionalization are needed. Finally, the use of aptamers affected NPs-cell interactions due to the formation of a corona coating [99], hence this approach was not useful to overcome the PEG dilemma. In conclusion, carboxymethyl dextran, hyaluronic acid and polymetacrylic acid may reduce the protein adsorption on the surface of CtNPs and may be taken for further evaluations.

Other approaches generated NPs based on chitosan in which the positive amino groups have been functionalised to reduce the adsorption of proteins. Chitosan derivatives include chitosan functionalised with IAA [70], ornithine [74], N-phosphorylcholine [75], N-octyl- N, O-carboxymethyl groups [6] and chitosan oligosaccharide [81] and were used to generate nanocarriers. NPs based on chitosan modified with IAA decreased the activation of the innate immune response by downregulating the activation of TLR 4 in macrophages [70]. However, further studies are needed to decrease their accumulation in the RES organs.

Moreover, NPs based on chitosan modified with ornithine were used for transfection studies as biomimetic nanocarrier [74]: in this study the adsorption of albumin was measured on the polymer, rather than on the formed nanocomplexes. Hence, further studies are needed to assess nanocomplex's behaviour in vivo. In another study, chitosan was modified with Pc to obtain a biomimetic nanocarrier: a lower protein adsorption on the formed NPs was recorded due to a lower positive ZP compared to NPs based on pristine chitosan [75]. However, an in vivo biodistribution investigation would help to assess the retention time in the blood. Conversely, micelles based on N-octyl-N, O-carboxymethyl chitosan underwent an in vivo biodistribution study which showed a low accumulation of micelles in the MPS organs, since their highly negative ZP reduced plasma proteins adsorption [6]. A study assessing the tumour targeting ability of those micelles following IV administration would help to move this chitosan derivative forward clinical investigations. Finally, chitosan oligosaccharide was used in conjunction with oleic acid to prepare self-assembled NPs which were found to adsorb albumin [81], but no effect of this adsorption was studied in cellular uptake investigations. In conclusion, these approaches using chitosan derivatives need further investigations in order to establish the derivative that give the best outcome.

Finally, some chitosan derivatives were used as stealth coating of other materials-based NPs. These derivatives include the negatively charged carboxymethyl chitosan [83], and the water soluble chitosan oligosaccharide [87,100] and succinic acid modified chitosan [89]. The negatively charged CmCt was used to coat liposomes, in turn coated with the positively charged trimethyl chitosan [83]. The protein adsorption was reduced compared to the control albeit biodistribution studies showed that the coated liposomes accumulated mainly in the RES organs suggesting their elimination. The hydrophilic chitosan oligosaccharide was used to coat the surface of PLGA NPs via formation of an amide bond. This induced lower protein adsorption than uncoated NPs, but still higher the PEG coating [87]. However, by using a dopamine polymerization method the surface of NPs was surrounded by a hard corona; the phagocytic uptake was reduced compared to PEG coating while an efficient cellular uptake by a tumour cell line was recorded regardless of the presence of the hard corona [100]. The only disadvantage of this method was the presence of IgG in the hard corona, known to be an opsonin, hence optimization must be performed before pursuing in vivo investigations. Finally, chitosan modified with succinic acid also known as "zwitterionic" polymer was used to coat the surface of several materials such as Eudragit E100 [89], PAMAM dendrimers [91], micelles [92] or nanogel [93]. In all these systems, zwitterionic chitosan was able to reduce protein adsorption and complement activation, along with enabling nanocarrier-cell interactions. Therefore, zwitterionic chitosan can be considered a safer coating than carboxymethyl chitosan and chitosan oligosaccharide, as also showed by the several applications illustrated by the literature.

Conclusion

CtNPs play a promising role in drug delivery, owing to chitosan's many advantageous features. However, its cationic nature in biological fluids causes an interaction with plasma proteins forming the so-called protein corona. This is responsible for rapid clearance of the nanocarriers by the immune system upon in vivo

intravenous administration. Despite the popularity of PEGylation, some key disadvantages such as the production of antibodies against the coated NPs and the poor cellular internalization limit its use; hence, several alternatives to PEGylation have gradually emerged in the literature. Some of these are based on the chemical modification of chitosan so to obtain biomimetic nanocarriers (e. g. ornithine modified chitosan) or to mask the positive amino charges (e. g. imidazole-acetic acid derivative), whereas others exploit the coating of CtNPs with polyanions (e. g. polyacrylic acid). Within all strategies, zwitterionic chitosan seems the most promising as stealth coating of nanocarriers. Perhaps the formation of protein corona might be investigated also in zwitterionic chitosan as backbone of nanocarriers, so to avoid a coating strategy. In any case, the aim is to modify the distribution of positive charges at the NPs' surface and, in turn, reduce the interaction with plasma proteins. This area of research is fast evolving, with numerous studies performed in vitro using FBS and very few studies assessing NPs' behaviour in in vivo models (such as mice). In addition, there is a general lack of research into analysing the behaviour of CtNPs upon incubation with human serum which better simulates the in vivo interaction of plasma proteins in human subjects. The efforts done by the literature to decrease the protein adsorption on CtNPs suggest that the relevant mucoadhesive property of chitosan is responsible for the formation of protein corona. This may indicate that the reasons lying on the use of chitosan to generate drug-load nanocarriers outmatch its mucoadhesive property, whilst giving value to its non-toxic, biocompatible and non-allergenic properties.

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Figure 1. Free positive amino groups on chitosan backbone result in the formation of positively charged nanoparticles characterized by the mucoadhesive property: the positive amino groups interact with the negatively charged cell membrane inducing endocytosis, hence adsorption of the nanocarrier.

Figure 2. Upon interaction with the biological milieu containing thousands of different proteins, nanoparticles (NPs) can adsorb either opsonins (such as complement proteins or Immunoglobulin G) or dysopsonins (such as Albumin or glycoproteins). The different proteins adsorption depends on the physico-chemical characteristics of NPs surface and leads to different fates of NPs. Opsonins adsorption determines NPs' macrophageal clearance through recognition of opsonins by scavenger receptors; instead, dysopsonins adsorption induces a prolonged NP's bioavailability.

Figure 3. In the biological milieu, a nanoparticle (NP) attracts negatively charged proteins such as albumin, which is highly abundant in the blood, forming the soft corona. Gradually, according to the Vroman effect, the soft corona may be replaced by other undefined proteins with higher molecular weight and stronger affinity forming the hard corona, which determines the fate of the NP.

Figure 4. Hydrophobicly modified hexanoyl-CtNPs (HCtNPs) and negatively charged succinoyl-CtNPs (SCtNPs) adsorb different amount of albumin upon incubation with 10% Fetal Bovine Serum (FBS), according to the type of modification: HCtNPs adsorb a higher amount of albumin on their surface comparing to SCtNPs. This phenomenon affects the size of NPs, increasing by the 20% for HCtNPs, while decreasing by the 30% for SCtNPs. As a result, a different degree of macrophage uptake is recorded: HCtNPs are phagocyted more efficiently than SCtNPs.

Figure 5. Schematic representation of the strategies adopted to decrease protein corona formation in chitosan-based nanoparticles (CtNP) and associated chemical structure of chitosan derivatives. Some chitosan derivatives were employed in the generation of CtNPs and these include chitosan modified with imidazole-acetic acid (IAA), ornithine and N-phosphorylcholine, N-octyl N, O-carboxylmethyl chitosan and chitosan oligosaccharide. Other chitosan derivatives were employed as stealth coating for different materials-based nanocarriers. These chitosan derivatives include carboxymethyl chitosan, oligomers and zwitterionic chitosan. The cationic surface of CtNPs was coated with several materials so to decrease the formation of protein corona and overcome PEGylation limitations. These coating materials include methoxylpolyethylene glycol (mPEG), carboxylated PEG (100) monostearate (PEGms) and D-tocopheryl PEG (1000) succinate (tPEGs). Polyanion materials include: carboxymethyl dextran, hyaluronic acid, alginate, polyacrylic acid and polymethacrylic acid. The use of aptamers was also investigated.

Figure 6. Representation of the “PEG dilemma”. Chitosan nanoparticle (CtNP) are loaded with a drug to form drug loaded CtNP. The nanocarrier is then coated with polyethylene glycol (PEG) by adsorption or covalent bound in a process known as PEGylation. The PEGylated CtNP is then injected into a mouse. Following in vivo administration, PEGylated CtNP protects the drug from enzymatic degradation. However, anti-PEG antibodies may be produced and the cellular interaction may be reduced, resulting in a poor drug’s therapeutic efficiency.